PREPARATION OF CHITOSAN-POLYVINYLPYRROLIDONE MICROSPHERES AND FILMS FOR CONTROLLED RELEASE AND TARGETING OF 5-FLUOROURACIL

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TAYLAN ÖZERKAN

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PREPARATION OF CHITOSAN-POLYVINYLPYRROLIDONE MICROSPHERES AND FILMS FOR CONTROLLED RELEASE AND TARGETING OF 5-FLUOROURACIL

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ABSTRACT

PREPARATION OF CHITOSAN-POLYVINYLPYRROLIDONE MICROSPHERES AND FILMS FOR CONTROLLED RELEASE AND TARGETING OF 5-FLUOROURACIL

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Controlled drug delivery systems deliver drugs at predetermined rates for extended periods. Although there are various types such as capsules, tablets etc, micro and nano spheres are the most commonly used systems. In this study, a set of chitosan-polyvinylpyrrolidone (CH-PVP) microspheres containing different amounts of polyvinylpyrrolidone as semi inter penetrating networks (semi-IPN) were prepared as controlled release systems. Emulsification method was applied for the preparation of microspheres and some of them were conjugated with a monoclonal antibody which is immunoglobulin G (IgG). CH-PVP films were also prepared by solvent casting method with the same composition as in the microspheres and, mechanical and surface properties of the films were examined. Prepared microspheres were characterized by SEM, stereo and confocal microscopes. Some microspheres were loaded with a model chemotherapeutic drug, 5-Fluorouracil (5-FU), and in-vitro release of 5-FU were examined in phosphate buffer solutions (pH 7.4, 0.01 M.) It was shown that for semi-IPN samples release was faster compared to pure CH samples and the total release was achived 30 days for CH:PVP-2:1, CH:PVP-3:1 semi-IPNs and CH microspheres

and 27 days for CH:PVP-1:1 semi-IPN microspheres. The antibody conjugated microspheres were targeted to MDA-MB (human causasian breast carcinoma cancer cells and coculture cells in culture medium. For the CH-PVP films, it was obtained that as the amount of PVP increased, hydrophobicity as well as mechanical strength of the system was decreased.

Keywords: Microspheres, controlled release, chitosan, targeting.

ÖZ

KONTROLLU SALIM İÇİN KİTOSAN-POLİVİNİLPİROLİDON MİKROKÜRE VE FİLMLERİN HAZIRLANMASI VE 5-FLOROURSİL'İN HEDEFLENMESİ

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Kontrollü ilaç salım sistemleri uzatılmış periyotlar için ilacı önceden belirlenmiş hızlarda salan sistemlerdir. Kapsül ve tablet gibi değişik tipleri olmasına rağmen mikro ve nanoküreler en çok kullanılan sistemlerdir. Bu çalışmada yarıyarıya-içiçe geçmiş ağ yapı (semi-IPN) şeklinde farklı miktarlarda polivinilpirolidon içeren kitosan-polivinilpirolidon (CH-PVP) mikroküreleri kontrollü salım sistemi olarak hazırlanmıştır. Mikrokürelerin hazırlanması için emülsiyon metodu kullanılmıştır ve mikrokürelerin bir kısmı immünoglobulin G monoklonal antikoru ile konjuge edilmiştir. Ayrıca mikrokürelerle aynı kompozisyonları içeren CH-PVP filmleri solvent uçurma yöntemi ile hazırlanmış ve bu filmlerin mekanik ve yüzey özellikleri incelenmiştir. Hazırlanan mikroküreler SEM, konfokal ve steryo mikroskopları ile karakterize edilmiştir. Mikrokürelerin bir kısmı model kemoterapik ilaç, 5-Florourasil (5-FU), ile yüklenmiş ve fosfat tanpon çözeltisi (pH 7.4, 0.01 M) içinde 5-FU in-vitro salımı çalışılmıştır. Yarıyarıya içiçe geçmiş ağ yapıdan yapılan salımın sadece kitosan ile hazırlanmış örneklerden daha hızlı olduğu ve toplam salımın CH:PVP-2:1 ve CH:PVP-3:1 semi-IPN örnekleri ile CH örneği için 30 günde, CH:PVP-1:1 semi-IPN örneği

içinse 27 günde tamamlandığı gösterilmiştir. CH-PVP filmleri için, PVP miktarı arttıkça mukavemet ve hidrofobik özelliklerin azaldığı gözlemlenmiştir.

Anahtar Kelimeler: Mikroküre, kontrollü salım, kitosan, hedefleme.

To My Dear Family,

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LIST OF SYMBOLS AND ABBREVIATIONS

CH	Chitosan
PVP	Polyvinylpyrrolidone
CH-PVP	Chitosan-polyvinylpyrrolidone
GA	Glutaraldehyde
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
NHS	N-hydroxyl succinimide
PBS	Phosphate buffer solution
DDSs	Drug delivery systems
CDDS	Controlled drug delivery systems
TDDSs	Targeted drug delivery systems
MAB-7	Human breast adenocarcinoma cell line
MDA-MB	Human causasian breast carcinoma cell line
MS	Microsphere
IPN	Inter penetrated network structure
GAGs	Glycosaminoglycans
AIBN	Azobisisobutyronitril
MAb	Monoclonal antibody
BsMAb	Bispecific mooconal antibodies
H	Heavy chain
L	Light chain
Fv	Variable region
Fc	Constant portion
IgG	Immunoglobulin G
5-FU	5-fluorouracil
SEM	Scanning electron microscopy
UTS	Ultimate tensile strength
E	Modulus of elasticity

SAB	Strain at break
FBS	Fetal bovine serum
RPMI 1640	Cell culture medium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

CHAPTER 1

INTRODUCTION

1.1 Controlled Drug Delivery Systems

In the last 20 years, a synergy among chemists, biologists, medical doctors, and engineers in biomedical materials is developed. Today we replace the materials in to the body so that provide significant therapeutic improvements and healing of diseased or damaged tissues are provided. As a result, patient outcomes have been vastly improved in diverse areas as cancers or severe burns [1]. Biomaterial can be defined as “the material which is used to replace part of a living system which is not functioning properly or to help the biological system in intimate contact with living tissue”. The most important biomedical applications of biodegradable polymers are in the areas of controlled drug delivery systems (DDSs) [2-4], implants and devices for hard and soft tissue repairs [5-7], surgical dressings [8], dental repairs, artificial heart valves, contact lenses, cardiac pacemakers, vascular grafts, organ regeneration and so on [9]. The biocompatibility and immunocompatibility of polymeric materials is of fundamental importance for their possible therapeutic uses. Soluble polymeric materials are mostly used as carriers of drugs, hormones, growth factors, enzymes and other active substances [10].

Controlled drug delivery systems are designed to deliver drugs at predetermined rates for predefined periods of time [11]. Ideally, a drug delivery system releases the drug in the right body compartment at the rate required for a specific treatment. The goal of controlled drug delivery systems is to deliver crucial

chemotherapeutic drugs to the tumor sites. In the meantime, controling of the delivery speed of the drug reduces the harm to other healty tissues [12]. Most available drug delivery systems are made of biodegradable, biocompatible and natural biopolymers and are capable of rate and/or time controlled drug release [13]. Controlled drug delivery systems may be classified into two general concepts; one is targeting, the other is controlled release. Systems delivering active agent only to the desired tissues and organs are called as targeted delivery systems (TDDSs) and systems only controlling the release rate of active agent are called as controlled drug delivery systems (CDDSs) [2]. In the 1950s controlled release systems were appeared and were originally used for administration of nonmedical agents, such as antifouling substances and pesticides in agricultural applications. After a decade they were introduced to medical researches and in the 1970s, slow release systems of large molecules like proteins were developed. Polymers obtained from lactic acid were used in the 1970s as the earliest drug delivery systems. Since polymers have the ability to be processed easily, they are still the most widely preferred materials for the design of drug delivery systems. In addition, chemical and physical properties of polymers can be easily controlled in the desired form during the molecular synthesis [3].

There are various forms of controlled release formulations which are shown in Figure 1.1.

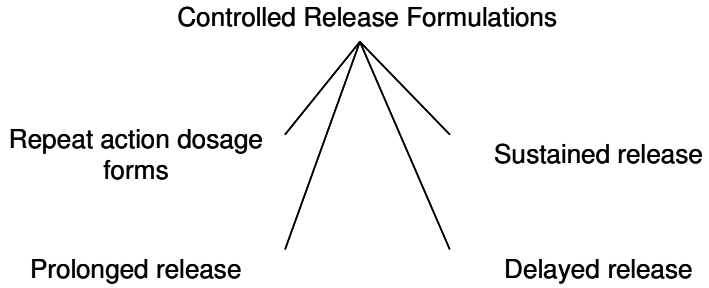


Figure 1.1 Controlled release formulations

Sustained-release systems provide effective concentrations for a long period of time. Such systems reduce dosing frequency, and thus, not only improve treatment compliance, but also reduce the necessity for frequent clinic visits [14].

In prolonged release systems in order to reduce toxic effects and to maintain therapeutic activity, the rate of release of active substance from the formulation after administration has been reduced, in order to maintain therapeutic activity [6].

In delayed release products; release of active substance is delayed for a finite "lag time", after which release is unhindered. For example enteric coated or gastro resistant oral capsules which remain intact in the stomach and only disintegrate in the higher pH of the small intestine.

A repeat action dosage form is designed to release initially the equivalent of a usual single dose of drug. Then, after a certain period another single dose of the drug is released [7].

1.1.1 CDDSs versus Conventional Systems

The ways in which chemicals or drugs are administered have gained increasing attention in the past two decades. In conventional systems, a chemical is

administered in a high dose at a given time and the same dose is repeated after several hours or days. This is not economical and sometimes results in damaging side effects. As a consequence, increasing attention has been focused on methods of giving drugs continuously for prolonged time periods and in a controlled fashion. The primary method of accomplishing this controlled release has been through incorporating the chemicals within polymers [15].

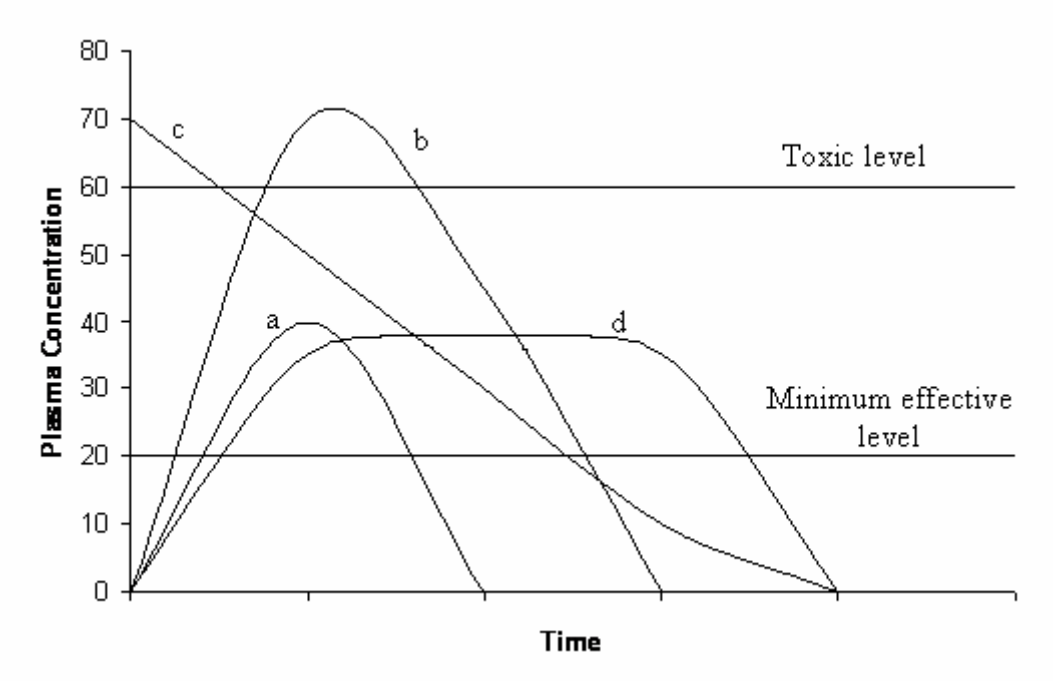


Figure 1.2 Plasma concentration of drug versus time profiles (a) Standard oral dose (b) oral overdose (c) intravenious injection (d) controlled release idael dose

As can be seen in Figure 1.2, the conventional oral and intravenous routes of drug administration do not provide ideal pharmacokinetic profiles especially for drugs, which display high toxicity or narrow therapeutic intervals. For such drugs the ideal pharmacokinetic profile will be the profile where the drug concentration is in

between therapeutic levels without exceeding the toxic level and minimum effective level without going down this level. Drug concentration maintains for extended periods of time between these levels till the desired therapeutic effect is reached [16].

1.1.2 Controlled Release Mechanisms

The continuous release of drugs from the polymer matrix could occur either by diffusion of the drug from the polymer matrix, or by the erosion of the polymer (due to degradation) or by a combination of these two mechanisms. For a given drug, the release kinetics from the polymer matrix are governed predominantly by three factors which are the polymer type, polymer morphology and the excipients present in the system [17].

The physical process of dissolution of a polymeric matrix or microsphere, in which the solid material slowly losses mass and eventually disappears, is called bioerosion. The mechanism of bioerosion may be simple: e. g., the solid polymer may erode by dissolution of the individual polymer chains exist in the matrix. In many cases the polymer chains within the matrix change in structure and permit bioerosion. For example, the molecular weight of the polymer may decrease within the matrix following placement within the biological environment. This process, called biodegradation, may occur enzymatically, relying on catalysts present within the environment or embedded within the polymer itself, or hydrolytically, if polymers that are susceptible to hydrolytic breakdown are used. As biodegradation proceeds, the molecular weight of the polymer decreases. When the constituent polymer molecules become sufficiently small, they dissolve and the polymer matrix erodes completely [18,19].

The drug will be released over time either by diffusion out of the polymer matrix or by degradation of the polymer backbone. This continuous release of the drug

could potentially lead to a pharmacokinetic profile close to the ideal case scenario shown in Figure 1.2.

1.1.3 Advantages of Polymeric Drug Delivery

There are three key advantages that polymeric drug delivery products can offer. These are summerized below;

Sustained delivery of drugs: Encapsulated drug is released over extended periods and hence eliminates the need for multiple introduction of drug doses. This feature can improve patient compliance especially for drugs for chronic indications, requiring frequent injections (such as for deficiency of certain proteins) [14].

Localized delivery of drugs: The product can be implanted directly at the site where drug action is needed and hence systemic exposure of the drug can be reduced. This becomes especially important for toxic drugs which are related to various sytemic side effects (such as the chemotherapeutic drugs).

Stabilization of the drug: The polymer can protect the drug from the physiological environment and hence improve its stability *in vivo*. This particular feature makes this technology attractive for the delivery of labile drugs such as proteins [16, 20].

1.1.4 Particulate DDSs

While implantable drug delivery systems may be useful for certain applications, in many cases an injectable or ingestable delivery system is desired. For that reason, polymer particulates represent a potentially important class of drug delivery systems. Because of their small size, polymer particulates can be injected into the desired tissue site or into the blood stream. Several different classes of particulates have been examined to use as drug delivery vehicles including microcapsules,

microspheres, and nanospheres. Microcapsules consist of a polymer shell enclosing a drug-loaded core. Microspheres are homogeneous particles in which the drug of interest is dispersed or dissolved within the solid polymer phase. Nanospheres (which are about 0,2-0,5 μm in diameter) are much smaller then microcapsules or microspheres (which are about 30-200 μm in diameter) [17]. Nanospheres are usually homogeneous particles, similar to microspheres, but they are frequently modified at the surface to increase their stability in the body or to provide targeting capability. A variety of methods have been reported for producing microspheres including phase separation by polymer/ polymer incompatibility and coacervation [21]; solvent evaporation or solvent removal [22]; hot-melt microencapsulation; spray drying; interfacial polymerization; and supercritical fluidprocessing techniques (such as the gas antisolvent spray precipitation process [23] or rapid expansion of supercritical fluids [24].

The rate of agent release from microsphere system depends on an intimidating number of variables including characteristics of the drug (size and solubility), properties of the polymer (composition and molecular weight), and the method of particle production (emulsion or suspension). The rate of release can usually be adjusted by changing these parameters.

1.1.4.1 Microspheres

An appropriate selection of the polymer matrix is necessary in order to develop a successful drug delivery system. The polymer could be non-degradable or degradable. A major disadvantage with non-degradable polymers is that a surgery is required to harvest these polymers out of the body once they are depleted of the drug. Hence, non-degradable polymers can be used only if removal of the implant is easy (such as ocular or vaginal implants). Degradable polymers on the other hand do not require surgical removal and hence are preferred for drug delivery applications. However, since they degrade to smaller absorbable molecules, it is important to make sure that the degraded metabolites are non-toxic in nature [24].

Biodegradable microparticles have been extensively employed as pharmaceutical formulations for many routes of drug delivery. In contrast to other carriers, polymeric microspheres are stable enough to permit their administration by the topical, oral or parenteral route [25]. In general, microparticles offer a number of advantages with respect to other delivery systems since (a) their physicochemical characteristics remain unaltered for long periods allowing long-term storage, (b) depending on their composition, they can be administered by different routes (e. g., oral, oral mucosal, intramuscular, or subcutaneous), (c) they protect encapsulated drug from enzymatic- or pH-dependant degradation, (d) they are suitable for industrial production, and (e) microsphere-based systems can be formulated to provide a constant drug concentration in the blood or to target the drug to specific cells or organs [26, 27]. Microspheres can also be used to treat diseases that require a sustained concentration of the drug at a particular anatomical site, e. g., the periodontal pocket. In this regard, the relationship between anatomical site and microparticle size should be considered. For instance, microspheres with diameters in the range of 20-120 μm can be utilized for oral, topical, subcutaneous, and periodontal pocket administration, since they are retained in the interstitial tissue and act as sustained-release depots. In contrast, smaller microparticles need to be prepared for application to other sites such as the eye, lung, and joints [28].

1.2 Release Kinetics

Many controlled-release products are designed on the principle of embedding the drug in a porous matrix. Liquid penetrates the matrix and dissolves the drug, which then diffuses into the exterior liquid. Higuchi tried to relate the drug release rate to the physical constants based on simple laws of diffusion. Release rate from both a planar surface and a sphere was considered. The analysis suggested that in the case of spherical pellets, the time required to release 50% of the drug was normally expected to be 10% of the time required to dissolve the last trace of solid drug in the center of the pellet.

Higuchi was the first to derive an equation to describe the release of a drug from an insoluble matrix as the square root of a time-dependent process based on Fickian diffusion. This equation is:

$$M_t/M_0 = Kt^{1/2} \quad (1)$$

where M_t is amount of drug release at time t, M_0 is the total amount, t is time and K is diffusion constant [29].

Release data can also be fitted to the well-known exponential equation (Korsmeyer equation), which is often used to describe the drug release behavior from polymeric systems:

$$\text{Log } (M\ t/M_f) = \text{Log } k + n \text{ Log } t \quad (2)$$

where, M_t is the amount of drug release at time t; M_f is the amount of drug release after infinite time; k is a release rate constant incorporating structural and geometric characteristics of the tablet; and *n* is the diffusional exponent indicative of the mechanism of drug release [30].

The other rate equations that can be applied to release data are zeroth order rate equation (3) and first order rate (4) equations:

$$M_t = M - kt \quad (3)$$

$$M_t = M\ e^{-kt} \quad (4)$$

where, M_t is the amount of drug release at time t, M is total amount of drug and k is diffusion constant.

1.3 Interpenetrating Network Structures

An interpenetrated network (IPN) is an assembly of two cross-linked polymers, at least one of which is synthesized and cross-linked in the presence of the other. If

only one component of the assembly is cross-linked leaving the other in linear form, the system is called semi-IPN [31].

The three conditions for eligibility as an IPN are: (1) the two polymers are synthesized and/or crosslinked in the presence of the other, (2) the two polymers have similar kinetics, and (3) the two polymers are not dramatically phase separated. Several kinds of IPN structures exist (See Figure 1.3).

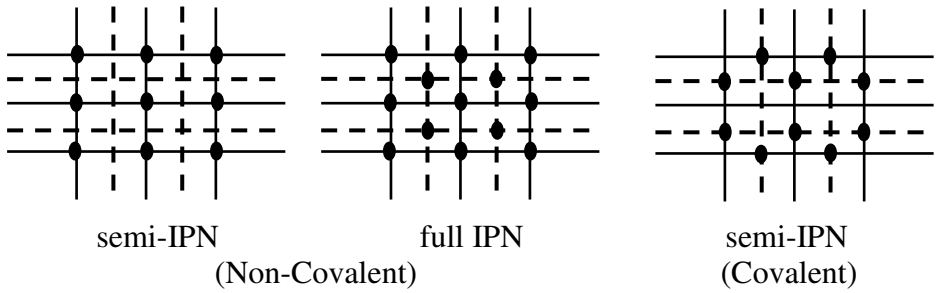


Figure 1.3 Inter penetrated network structures

These systems differ mainly because of the number and types of crosslinks that exist in the system. A non-covalent semi-IPN is one in which only one of the polymer systems is crosslinked [32]. For non-covalent semi-IPN and full IPN structures, two polymers are not bound to each other chemically but in the covalent semi-IPN structure two polymers are bound to each other.

1.4 Chitosan

Chitosan is partially or fully deacetylated derivative of chitin, the primary structural polymer in arthropod exoskeletons. From the biomaterials standpoint, chitosans are the most promising polysaccharides, with great potential for

development of resorbable and biologically active implant materials [33-35]. The primary source for chitin is shells from crab, shrimp, and lobster. Shells are ground, demineralized with HCl, deproteinized with a protease or dilute NaOH, and then deacetylated with concentrated NaOH. Structurally, chitosans are very similar to cellulose. The polymer is linear, consisting of β(1,4) linked D-glucosamine residues with a variable number of randomly located N-acetyl-glucosamine groups with different molecular weights and degree of deacetylation [36]. In essence, chitosan is cellulose with the 2-hydroxyl group replaced by an amino or acetylated amino group (Figure1.4).

Chitosan can be considered as belonging to the family of glycosaminoglycans (GAGs), a family to which also belong chondroitin sulfates, hyaluronic acid, and heparine.Glycosaminoglycans are particularly interesting since they seem to be alone among the polysaccharides that express the property of bioactivity [37].

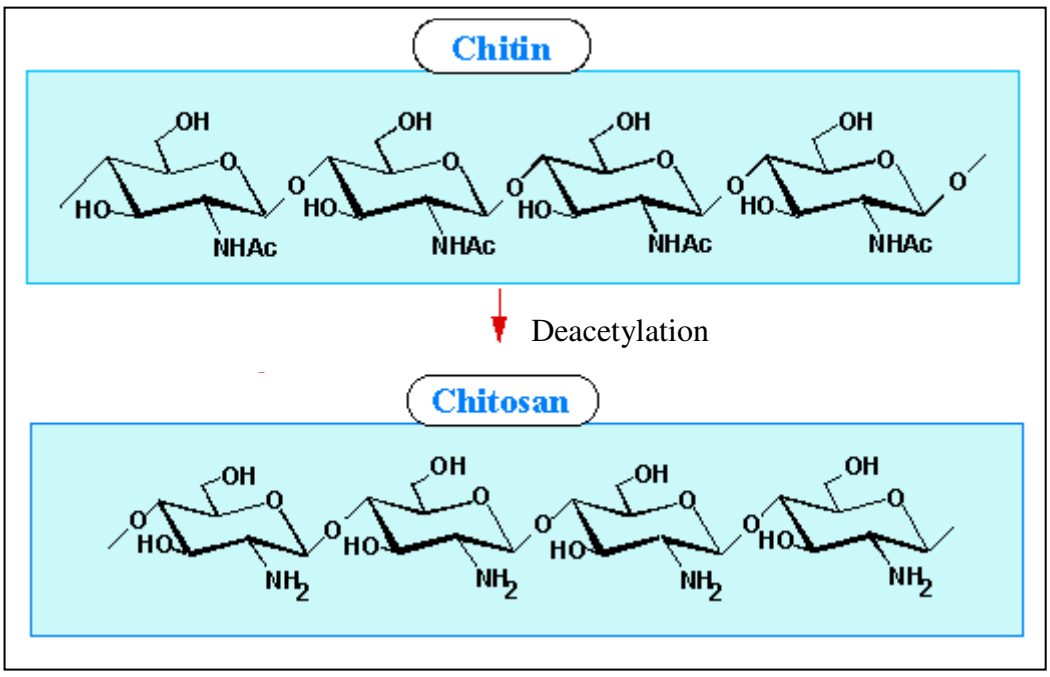


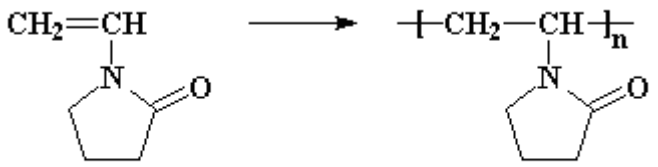
Figure 1.4 Structure of chitin and chitosan

This polymer is of great importance for many applications in agriculture, water purification, biomedical application, and cosmetics. Chitin and chitosan are good film-forming polymers; in addition chitosan is a chelating polymer and also the only cationic pseudo natural polysaccharide. It can be used as thickener, suspending agent, flocculating polymer to recover proteins, to concentrate dispersed particules or to purify water [38] and in controlled drug release, fast release dosage forms, peptide delivery and as adsorption enhancer for hydrophilic drugs [36].

1.5 Polyvinylpyrrolidone

Polyvinylpyrrolidone (PVP) was patented in 1939 and initially used in blood plasma during the Second World War before being used more widely in the pharmaceutical industry. It has been used in personal care products since 1950s and for many years has been used as a food additive and also has been used extensively in the formulations of pharmaceutical systems [36-39].

Poly vinylpyrrolidone is a vinylic polymer. It is synthetized via a radical polymerisation reaction starting from the vinylpyrrolidone monomer, using an initiator such as Azobisisobutyronitrile (AIBN) (Figure 1.5) [40].



vinylpyrrolidone polyvinylpyrrolidon

Figure 1.5 Structure of vinylpyrrolidone an polyvinylpyrrolidone

Vinyl polymers, with few exceptions, are generally not susceptible to hydrolysis. Polymers with main chains having only carbon-carbon bonds (with few exceptions) are not suspectible to enzyme-catalyzed reactions. Their biodegradation requires an oxidation process, and most of the biodegradable vinyl polymers contain an easily oxidizable functional group [41].

According to carcinogenecity studies it is proved that administration of PVP to rats and rabbits showed very little or no carcinomas [42-44]. The only chronic toxic effect noted in man upon subcutaneous injection has been cutaneous thesaurismosis after parenteral doses of 200-1000 g over 3-12 years [45].

1.6 Drug Targeting

One of the most important goals of pharmaceutical research and development is targeted drug delivery, defined as optimization of the therapeutic index by localizing the pharmacological activity of the drug to the site of action [46, 47] (Figure.1.6). It is important to distinguish this broad definition from a narrower definition of the basic targeting concept. Within this narrower definition, a specific drug receptor is considered as target, and the aim is to improve fitting and binding to this receptor that ultimately will trigger the pharmacological activity. Ever since the development of the receptor theory, attempts have been directed toward developing new therapeutic agents that have a singular target, that is, agents that bind only to a specific receptor. It was hoped that this way any toxicity would be avoided, and only the desired therapeutic gain would be produced. Unfortunately, the situation is not so simple. Most highly active new therapeutic agents designed to bind to a specific receptor ultimately had to be discarded when unacceptable toxicity or unavoidable side effects were encountered in later stages of the development.

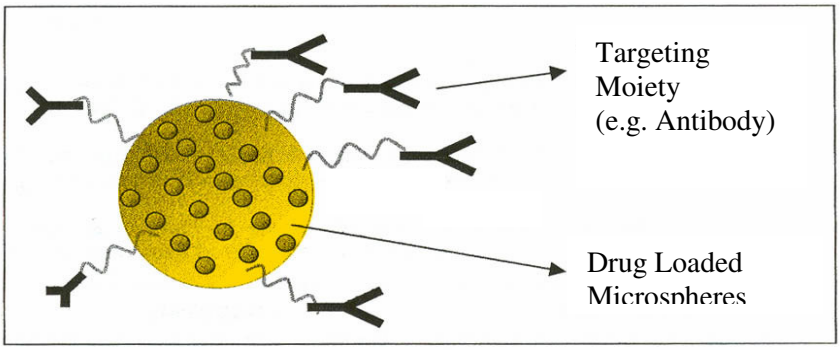


Figure 1.6 Shematic representation of targeting

Beyond receptor targeting, something additional has to be done, one needs to localize drugs at the desired site of action. Successful targeting, meaning preferential delivery, would lead to reduced drug dosage, decreased toxicity, and increased treatment efficacy. With reasonable biological activity, targeting to the site of action should be superior to molecular manipulations which aim receptor-substrate interactions. However, successful drug targeting is a complicated problem, because any drug introduced into the body encounters or must pass various organs, cells, membranes, enzymes, and receptors before reaching its designated target. During the past two decades, significant efforts have been focused on the field of site-specific, targeted drug delivery systems [46].

1.6.1 Currently Available Therapeutics

Clinical cancer chemotherapy in the 20th century has been dominated by the development of genotoxic drugs, initiated in 1940's by the discovery of the anticancer properties of nitrogen mustard and the folic acid analogue aminopterin. It is difficult to assign a date to the beginning of the treatment of cancer with drugs because herbal and other preparations have been used for cancer treatment since antiquity.

However, the 1890s, a decade that represents an extraordinarily creative period in painting, music, literature, and technology, encompassed discoveries that were to set the scene for developments in cancer treatment in the 20th century [48].

Chemotherapy is one of the major treatments in cancer therapy. However, it is often associated with severe side effects due to the fact that anticancer drugs are primarily cytotoxic agents that not only kill cancer cells but also cause damage to normal cells, especially proliferating cells such as bone marrow and gut epithelia cells. As a result, the success of chemotherapy is often hampered by the severe systemic side effects of chemotherapeutic drugs. Consequently, increasing the selectivity of the chemotherapeutic agents has gain a great importance to improve chemotherapeutic efficacy. There are three general approaches in increasing the selectivity: (1) identify agents that will be more selective in killing cancer cells than normal cells; (2) deliver the chemotherapeutic agent more selectively (ideally, specifically) to cancer cells; and (3) mask the chemotherapeutic agent in such a way that it will be released selectively in cancer cells [47].

Non-surgical methods of cancer treatment, primarily radiation and chemotherapy, rely on procedures that kill cells. The main problem with these treatments is that they do not provide specificity for cancer cells. In the case of radiaton therapy, a degree of specificity is achived by localizing the radiation to the tumour and its immediate surrounding normal tissue. For anti-cancer drugs, it is primarily the rapid proliferation of many of the cancer cells that makes them more sensitive to cell killing than their normal counterparts. However, both cases are limited by their cytotoxic effects on normal cells. In the case of radiotherapy, normal tissue surrounding the tumour limits the radiation dose, whereas for anti-cancer drugs, it is usually the killing of rapidly dividing normal cells such as in the bone marrow, hair follicles and epithelial cells lining the gastriointestinal tract, which limits the dose that can be applied to the patient [49].

1.6.2 Chemotherapy Optimization

The current concept that chemotherapeutic agents are administered at a dose to the maximum a patient can tolerate (before the onset of unacceptable toxicity) is in wide clinical use today. This approach is based on a series of analyses which indicated that the greater the dose intensity of an anticancer drug, the better the outcome. However, as indicated, the therapeutic range for most anticancer agents is extremely narrow, and in most cases no information is available on the sensitivity of a patient's tumor to a particular agent and the patient's tolerability of a given dose prior to therapy. Hence, the dosage of chemotherapeutic agents remains largely empirical and is basically derived from the kind of information derived from in-situ experiments and in-vivo applications.
Chemotherapy drugs are sometimes feared because of a patient's concern about toxic effects. Their role is to slow and hopefully halt the growth and spread of a cancer. There are three goals associated with the use of the most commonly-used anticancer agents which are (1) damage the DNA of the affected cancer cells, (2) inhibit the synthesis of new DNA strands to stop the cell from replicating, because the replication of the cell is what allows the tumor to grow (3) stop mitosis or the actual splitting of the original cell into two new cells. Stopping mitosis stops cell division (replication) of the cancer and may ultimately halt the progression of the cancer [50].

Commonly used chemotherapy agents in drug delivery systems are, Methotrexate [51], Dexamethasone [52], Doxorubicin [53], Camptothecin[54], Mitomycin[55], Taxol[56] and 5-Fluorouracil (130.077 g/mol) [57].

The chemotherapy agent 5-Fluorouracil (5-FU, 5-fluoro-1H-pyrimidine-2,4-dione) (Figure 1.7), which has been in use against cancer for about 40 years, acts in several ways, but principally as a thymidylate synthase inhibitor, interrupting the action of an enzyme which is a critical factor in the synthesis of pyrimidine-which is important in DNA replication. Some of its principal use is in colorectal cancer

and pancreatic cancer, in which it has been the established form of chemotherapy for decades. Side effects include myelosuppression, mucositis, dermatitis, diarrhea and cardiac toxicity [58].

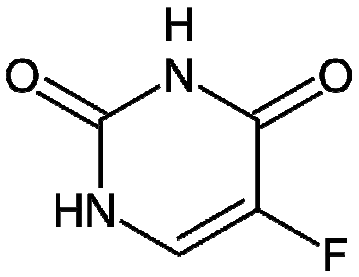


Figure 1.7 Chemical structure of 5-Fluorouracil

1.6.3 Strategies to Deliver the Drugs to Targets

Some approaches have been done in attempting to increase the therapeutic index by improving the specificity and efficacy of the drug and reducing the toxicity. One example of this is to target the cytotoxic agent to the tumour cells. To increase the specificity and reduce toxicity, triggered mechanisms have been designed to activate cytotoxic agents synthesized in their pro-drug or inactive forms, in a site selective manner [59]. Triggering signals can be either light or chemicals or cellular factors such as enzymes. The inherent features of cancer cells can also be used in the development of targetting agents for tumour cells. Cancer cells often over-express specific tumour antigens, carbohydrate structures, or growth factor receptors on their cell surface. In addition to tumour cell membrane-specific antigens, some cells also express unique protease [60]. Based on the above concepts, various strategies for targetting cytotoxic agenst are under development and are currently being tested in pre-clinical or clinical settings.

These include: (1) monoclonal antibodies (MAbs) against tumour-associated antigens [59], (2) bispecific monoclonal antibodies (BsMAbs) which combine the specificity of two different antibodies within one molecule and cross-link an effector cell or a toxic molecule with the target cell, (3) pro-drugs in conjuction with enzymes or enzyme-Mab conjugates [60], (4) synthetic copolymers as drug carriers, (5) liposomes as carriers for drug delivery.

1.6.3.1 MAb Mediated Therapeutics

Antibodies are protein complexes produced by B lymphocytes (B cells). An antibody consists of two heavy and two light peptide chains which are expressed as H and L, respectively. The heavy and large chains are held together by disulfide bonds and covalent interactions. Antibodies have two important domains which are the variable region (Fv) and the constant portion (Fc). Fv contains the antigen binding end of the antibody and Fc interacts with complement or phagocytic cells via a glycosylated region of the protein fragments [63, 64].
The most common antibody is immunoglobulin G (IgG) which is shown in Figure 1.8. IgG molecules are Y-shaped proteins having H and L. The hinge region is connected via disulfide bonds [64].

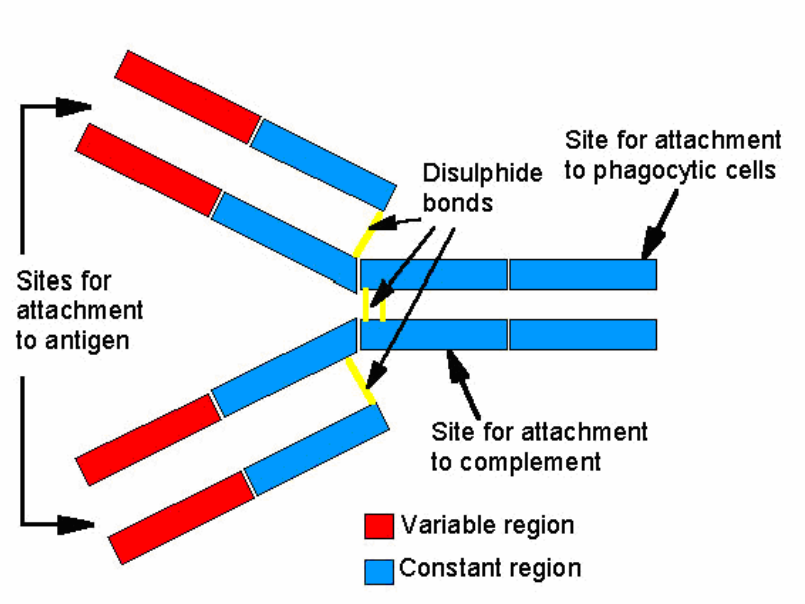


Figure 1.8 Schematic representation of an IgG molecule

The development of anti-bodies gave rise to the development of antibody-mediated therapeutics for cancer [65]. Since they have a unique specificity, MAbs were tought that they will become very important in cancer therapeutics. Over the last two decades the usage of MAbs introduced to clinical studies of malignancies. They have been coupled to drugs, toxins, enzymes, radionuclides, cytokines and drug-filled liposomes.

1.6.3.2 Antigenic Targets

An antigen can be defined as any material that the body recognizes as foreign substance. Such as nonhuman animal proteins, macromolecules on organs from a noncompatible donor and the pollens of some plants [66]. A vast number of new antigen targets are rapidly being discovered to sequence the human genome and to identify the expression of all genes in cancer cells.

Once the specificity of expression in tumor is determined, the antigens can serve as targets for antibody-based therapeutics. Techniques to generate fully human antibodies are now readily available; therefore, many new antibodies or antibody-based constructs are likely to be introduced into clinical trials in the near future. However, the mechanisms by which the antibodies mediate antitumor activity are not fully understood, and the issues of which antibodies to select for clinical trials, and what approaches are optimal for antitumor activity, have not been resolved. The existence of tumor-specific or tumor-associated antigens is now well established. Furthermore, in animal tumor models and in patients, there is substantial evidence that immune responses directed against tumor antigens can cause the regression of established tumors. Theoretically, any cell surface molecule associated with tumor cells, tumor stroma, or tumor vasculature is a potential target for an antibody-based therapeutic approach (Figure 1.9). Furthermore, antibodies may be used to reduce or block growth factors that are directly or indirectly supporting tumor cell growth or the formation of tumor vasculature [67].

The choice of the target antigen is of great importance. Some considerations need to be taken which are, (1) The antigen on the tumour cell should be homogenous throughout the tumour and high enough to ensure the effective binding or the antibody, (2) antigen on the normal tissues should be limited or if the antigen is expressed on normal tissue, it should be inaccessible to antibodies in these tissues, (3) the antigen should be membrane bound and should not leave the cell surface.

Another approach is to target antibodies to antigens expressed on the tumour vasculature rather than to tumour-associated antigens. This is studied in pre-clinical models [68]. Directing to the accessible vascular compartment reduces the impact of the physical barriers of solid tumours, such as heterogeneous blood flow and elevated interstitial pressure [69].

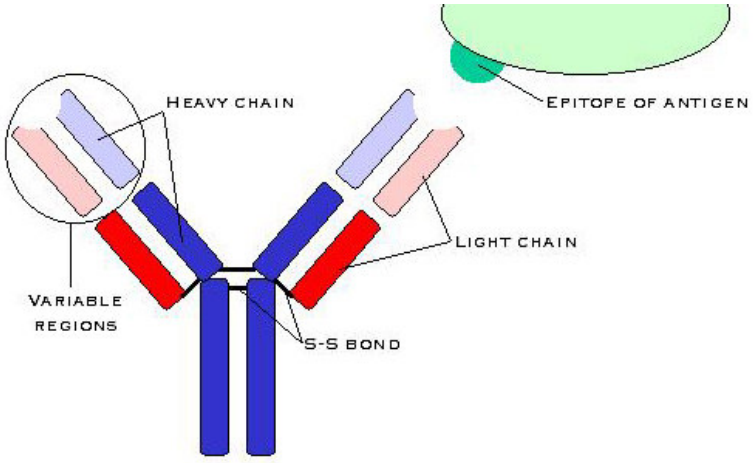


Figure 1.9 Schematic structure of an IgG and target

The selectivity of antigen-antibody interactions is analogous to the selectivity of substrate-enzyme interactions. The antigen-binding site of an antibody has a structure that allows a complementary fit with structural elements and functional groups on the antigen. The portion of the antigen that interacts specifically with the antigen-binding site on the antibody is called the antigenic determinant, or epitope [70].

1.6.3.3 Site-Specific Delivery

Placement of a biomaterial construct, such as a wafer or microspheres, at a physiological site of action, such as diseased tissue, is referred to as sitespecific delivery. In many applications a localized approach to treatment is more efficient than a systemic approach. However, the pinnacle of therapeutic treatment is to simply have a patient swallow a pill and design a therapeutic drug that targets a desired location or disease path. The effort to create targeted delivery has as one of its major goals the identification of specific antigen-antibody pairs that direct therapeutic uptake or dictate cell behavior [71].

1.6.3.4 Conjugation

Immobilized enzymes have recently became an important part in medicine. This is due to the recognition that immobilized enzymes have much potential for basic research and for practical applications in many areas. Methods available for immobilization of enzymes or other proteins are classified into four basic approaches which are (1) covalently bound, (2) adsorbed, (3) matrix-entrapped, (4) microencapsulated. One of the most widely studied basic approach is covalent bonding method. This involves covalent linkage between the protein and functional groups of the polymer. The first examples were described by Grubhofer and Schleit in 1953. Since then, a great deal of work has been carried for bonding proteins to polymeric or non-polymeric systems.

Proteins can be bound to a water-insoluble natural carrier like starch, cellulose, chitosan and etc. This is achived by intermolecular cross-linking by using multifunctional reagents like glutaraldehyde or carbodiimides [72]. The following features of covalently bound proteins have important bearings on their fisibility for biomedical applications. It is possible to vary the ratio of the amounts of the protein to carrier in these systems. For instance, in the case of enzymes covalently bound to insoluble carriers, it could range from as low as microgram of enzyme per gram of carrier, to as high as 3.5 grams of enzyme per gram of carrier [73]. Another advantage is the ease of the process technique is simple.

One of the most important considerations of covalently bound proteins in biomedical applications is their stability. Covalent binding increases thermal and storage stability of proteins. However this is not a general rule, since some may have lower stability. The major disadvantage is that some enzymes are sensitive to the coupling reagents and lose activity in the process [74].

Because of all of these arguments, immuno conjugates of antibodies with toxic agents or carriers are becoming a significant component of anticancer treatments. One ongoing challenge to the success of this approach, as mentioned before, is the prevention of exposure of healthy tissues to the employed cytotoxic agents [75].

By this method drug loaded polymeric micro or nano particles can be conjugated with an antibody specific to cancer cells and can be directly to the tumour area.

1.7 Aim of the Study

The aim of this study was to prepare surface activated micro carriers for chemotherapeutic drugs to target the drugs to a specific tumour site with a controllable rate. For this purpose chitosan-polyvinylpyrrolidone (CH-PVP) microspheres were prepared in different compositions by water/oil emulsification method. The microspheres had semi-IPN structures in which chitosans were crosslinked with glutaraldehyde and PVP molecules were entrapped in the matrix. It was expected that, in aqueous media PVP would dissolve and diffuse out creating a passage for the drug molecules. Therefore microspheres with various CH/PVP ratios were prepared. A model chemoterapeutic drug, 5-fluorouracil (5-FU) was loaded into the microspheres during preparation step. Release kinetic of 5-FU from microspheres were studied in phosphate buffer (pH 7.4, 0.01 M) media. For targeting purpose microspheres were activated by conjugating with immunoglobulin G (IgG) and the effect of drug loaded microspheres on MCF-7 cancer cells were examined in-vitro conditions.

CHAPTER 2

EXPERIMENTAL

2.1 Materials

Chitosan (> 85% deacetylated) and polyvinyl-pyrrolidone (MW 10,000) were products of Sigma, USA. Acetic acid was obtained from J.T. Baker, Netherland. Glutaraldehyde was purchused from BDH Limited, England, and Tween 80 was purchused from Acros Organics, USA. Corn oil was supplied from Sayınlar A. Ş., Turkey. 5 Fluorouracil (5-FU) was obtained from Fellbach, Germany. Lysozome (activity 20000 u/mg) was purchused from Datex Applichem, Germany. IgG (2 mg/mL) was purchused from Jackson Immuno Research, USA. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxyl succinimide (NHS) were purchused fom Sigma, USA. MCF-7 (human breast adenocarcinoma) and MDA-MB (human causasian breast carcinoma) were obtained from HÜKÜK (Hücre Kültürü Kolleksiyonu) Şap Enstitüsü, Turkey. Both were breast carcinoma cell lines.

2.2 Instrumentation

Mechanical stirrer (Heidoplph RZR 2021, Germany) was used for the preparation of microspheres.

Size determination of microspheres was performed by Malvern Mastersizer (Malvern Instruments, MAN 0103, UK).

Samples were examined with Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy (Perkin Elmer Spectrum BX ATR-FTIR

system equipped with PIKE single reflection MIRacle ATR component). ATR crystal was dimond.

Physical appearances of the prepared samples were examined by Scanning Electron Microscope (SEM, JEOL JSM-6400, NORAN Instruments, Tokyo, Japan).

Amount of released drugs (5-FU) were detected spectrophotometrically by using Agilent 8453 UV-Vis spectrophotometer (USA) at 266 nm. Calibration curve is given in Appendix A. Cell absorbance was determined at 570 nm by using VersaMax UV-visible spectrophotometer multiplate reader (Molecular Device, USA).

Static contact angles were determined by goniometer (CAM 200, Finland) applying sessile drop method. System is a computer controlled and user programmable video based instrument equipped with a dispenser.

2.3 Experimental Procedure

2.3.1 Preparation of Microspheres

For the preparation of chitosan microspheres, 0,3 gram grinded chitosan was dissolved in 10 mL aqueous acetic acid solution (5% v/v) and 2 mL of GA solutions (1.25%, 2.50% and 5.00% v/v prepared from 50% v/v stock solution) were used as cross-linker for each solution separately (Table 2.1) Then this solutions were suspended in 50 mL corn oil with addition of 0.5 mL tween 80 and stirred at 1000 rpm for 30 minutes. While stirring upon 30 minutes, 1 mL of glutaraldehyde was added and after 15 minutes another 1 mL of glutaraldehyde was added. After five hours of stirring microspheres were filtered and washed with acetone to remove oil. Then microspheres were placed on a petri dish and left them to dry at 50^{o}C for 12 hours. After drying, microspheres were stored in a plastic bottle.

Table 2.1 Composition of GA solutions

Sample	**GA/water (v/v)**	**% of GA solutions (2 mL)**
CH-1.25	1.25 mL/98.75 mL	1.25%
CH-2.5	2.5 mL/97.25 mL	2.50%
CH-5	5.0 mL/95.00 mL	5.00%

Chitosan-polyvinylpyrrolidone (CH-PVP) microspheres with different compositions were prepared similarly applying the described procedure. Weighed amount of grinded chitosan was dissolved in 10 mL acetic acid (5% v/v) solution and weighed amount of polyvinylpyrrolidone was dissolved in 1mL distilled water. Then chitosan solution and PVP solution were combined and 1 mg of 5-FU was added. After the addition of drug, the solution was suspended in 50 mL corn oil with addition of 0.5 mL tween 80 and was stirred at 1000 rpm for 30 minutes. Upon 30 minutes stirring 1 mL of 5% GA was added and after 15 minutes of addition of 1 mL of 5% GA, another 1 mL of 5% GA was added stirring at room temperature. Compositions of microspheres are given in Table 2.2.

Table 2.2 Composition of CH and CH-PVP microspheres

Sample	**Composition (CH/PVP) (w/w)**
CH	0.3g/0.0g
CH:PVP-1:0,33	0.3g/0.1g
CH:PVP-1:0,5	0.3g/0.15g
CH:PVP-1:1	0.3g/0.3g

After 5 hours stirring, cross-linked microspheres were filtered via vacuum filtration and washed with acetone several times then dried in an oven at 50^{o}C for 12 hours. Two sets were prepared, weighed, sieved (mesh size 150 μm) and then stored to use in release experiments (Table 2.3). For release experiments 0.1 g of microspheres (MS) were used.

Table 2.3 Obtained and used amount of microspheres

Sample	Total MS obtained	MS used in release experiments	Added drug
CH	0,195 g	0,1 g	1000 μg
CH:PVP-1:1	0,412 g	0,1 g	1000 μg
CH:PVP-1:0,5	0,382 g	0,1 g	1000 μg
CH:PVP-1:0,33	0,267 g	0,1 g	1000 μg

The prepared microspheres were examined by SEM (Jed Model 6400) often coated with gold, and particle size analyzer (Malvern Mastersizer S version 2.15).

2.3.2 Preparation of CH-PVP Films

3% chitosan solution was used for the preparation of microspheres but this chitosan concentration was too viscous for casting to prepare proper films. For this reason, for film preparation, 1% chitosan solutions were used. Chitosan solutions were prepared by dissolving 200 mg chitosan in 20 mL of 5% aqueous acetic acid solution at ambient temperature with stirring. Then weighed amount of PVP (0.2 g, 0.1 g and 0.066 g) were dissolved in 2 mL distilled water and mixed with chitosan solutions seperately. As crosslinker, different concentrations of glutaraldehyde were added (Table 2.4). Pure chitosan films could not be prepared because of the shrinking and breaking of the films (Figure 2.1).

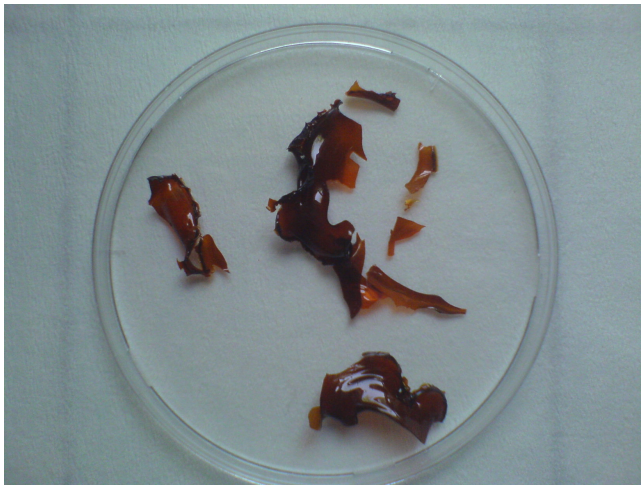


Figure 2.1 Shrunk chitosan film prepared by 5% GA

The concentrations of glutaraldehyde solutions were; 1%, 2.5%, 5.0% (v/v). 2 mL of each solution was added to 20 mL 1% (w/v) chitosan solution and stirred for 30 minutes and then the solutions were poured into plastic petri dishes (diameter = 9 cm). The films were obtained after evaporation of water at room temperature. The thickness of the films were detected by micrometer and found about 40±2 μm. The chemical structure of the films was examined by IR (Perkin Elmer Spectrum BX ATR-FTIR system).

Table 2.4 Compositions of CH-PVP semi-IPN films

Code	GA solution	CH/PVP (w/w)
CH:PVP-1:1-1	1.0%	0.2g/0.2g
CH:PVP-1:1-2.5	2.5%	
CH:PVP-1:1-5	5.0%	
CH:PVP-1:0,5-1	1.0%	0.2g/0.1g
CH:PVP-1:0,5-2.5	2.5%	
CH:PVP-1:0,5-5	5.0%	
CH:PVP-1:0,33-1	1.0%	0.2g/0.066g
CH:PVP-1:0,33-2.5	2.5%	
CH:PVP-1:0,33-5	5.0%	

2.3.3 Mechanical Tests

Tensile tests were applied to the prepared CH-PVP semi-IPN films crosslinked with different percent of GA solutions. The films were cut as rectangular strips and placed into the instrument. The gage length was 30±2 mm and width was 10 mm for each sample. The thickness of each specimen was measured by a micrometer at different parts including two ends and the middle. The average of these values was used in the calculations. At least five experiments were carried out for each type of films and average values were calculated.

LLOYD LRX 5K (LLOYD Instrument, ENGLAND), equipped with a 100 N load cell, was used for mechanical testing experiments (Figure 2.2). The mechanical test machine was under the control of a computer running program WindapR. During measurement, the film was pulled by top clamp at a rate of 3 mm/min. The tensile load applied on the specimen was continuously recorded by the computer.

Figure 2.2 Picture of the mechanical test instrument

The tensile strength for each specimen was obtained from the equation of $\rho=F/A$ where ρ is the tensile strength (in MPa), F is the maximum load (in N) applied just before rupture and A is the initial area (in mm^2) of the specimen. The load deformation curve was converted to stress-strain curve, where stress is the load per unit area (F/A as pascal) and strain is deformation per unit length ($\Delta l/l_0$, where l_0 is the initial length and Δl is the change in the length). Slope of the straight line exist in elastic region of the stress-strain curve is accepted as the elastic modulus (in GPa) of the specimen.

2.3.4 Contact Angle Measurement

Control and crosslinked CH-PVP semi-IPN film samples were used in contact angle measurements for the investigation of hydrophobicity-hydrophilicity variations of the CH-PVP matrices.

Contact angles of the samples were obtained by goniometer immediately after putting deionized distilled water droplets onto the polymer surfaces at room temperature. At least 5 measurements were obtained for each sample and average values were obtained. The goniometer instrument is given in Figure 2.3.

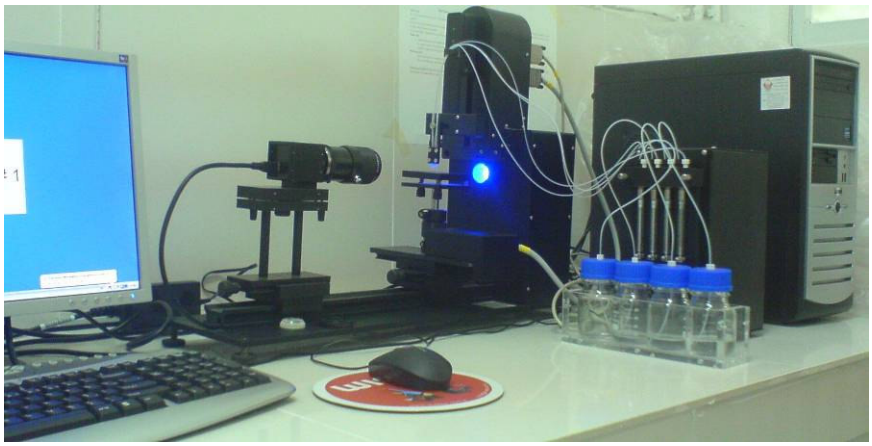


Figure 2.3 Goniometer used in contact angle measurements

2.3.5 In-vitro Release Studies

5-Fluorouracil release profiles from microspheres were obtained in-vitro by using dialysis method. For this purpose, 0.1 g microspheres, loaded with 5-Fluorouracil, were placed into a dialysis tube (molecular weight cut off 12000 D), then soaked in 10 mL of phosphate buffer solution (PBS, 0.01 M, pH 7.4) and put in a shaking water bath at 37°C. At every 24 hours intervals, the solution medium was withdrawn and immediately replaced with equal volumes of fresh PBS. The drawn solutions were analyzed spectrophotometrically at λ=266 nm in order to determine the amount of released 5-Fluorouracil by using a calibration curve (Appendix A).

2.3.6 Degradation of Microspheres

10 mg of microspheres were incubated in water bath at constant temperature of 37^{o}C in 10 mL PBS (0.01 M pH 7.4) containing 30 mg of lysozyme and these experiments were followed for 60 days in water bath. In certain periods little amount of samples were taken out to examine the changes in the physical shapes of microspheres by stereo microscopy and SEM. Additionally, SEM pictures of freeze dried CH and CH:PVP-1:1 microspheres were taken to observe the pores in the structure. For this purpose, some CH and CH:PVP-1:1 microspheres were put in to 10 mL PBS (0.01 M pH 7.4) solution for 60 days. After 2 days and 60 days some amount of microspheres were taken and dried in a freeze-drier for 1 day.

2.3.7 Conjugation of Microspheres

CH:PVP-1:1 microspheres crosslinked with 5% GA were chosen for IgG conjugation experiments. For this purpose 3 mg of microspheres were put in 0.5 mL PBS incubated overnight at +4^{o}C in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), immunoglobulin G (IgG) and N-hydroxyl succinimide (NHS). EDC (250 µL, from the stock solution of 2.5 mg/mL PBS), IgG (250 µL, from 10µL IgG in 240 µL PBS) and NHS (10µL, 0.92 mg/mL DMF) were mixed in 25:25:1 (v/v/v) ratio. After incubation of 24 hours, conjugated microspheres were washed with PBS (pH 7.4, 0,01M) solution in centrifuge for few minutes. Then microspheres left under vacuum to remove water.

2.3.8 Cell Culture Studies

In vitro cell studies were performed to investigate the effect of IgG conjugation on the microspheres. Either MCF-7 culture or MCF-7 and MDA-MB cocultures were used. So, the purpose of the coculture of MCF-7 and MDA-MB is to investigate the presence of specific activity of the IgG conjugated microspheres to the MCF-7

cells. These cells were chosen because MCF-7 cells have estrogen receptors but MDA-MB cells do not. Unloaded, drug loaded, conjugated drug loaded and conjugated unloaded CH:PVP-1:1 microspheres (crosslinked with 5% GA) were prepared for the cell culture and coculture experiments (Table 2.5). 8 samples from set A and 8 samples from set B, 1 sample from C and 1 sample from D were prepared.

Table 2.5 Prepared samples for cell culture experiments

Sample code	Sample content
F0	Control-Free drug
MSA	Unloaded microspheres
MSB	Drug loaded microspheres
MSC	Conjugated drug loaded microspheres
MSD	Unloaded conjugated microspheres

MCF-7 cell line was routinely cultivated in RPMI 1640 supplemented with 10% FBS (Fetal bovine serum), penicillin (100 U/mL) and streptomycin (100 mg/mL) at 37^{o}C, and 5% CO_2. 6x10^3 cells were seeded into each well of a 96-well plate and incubated for 24 h at 37^{o}C. Then, each well of the cell cultures were exposed to 100 µL of polymer test specimens (0,1 mg microsphere in 100 µL) . After, 144 h (6 days) incubation time and 240 h (10 days) incubation time, cells were microphotographed (in the wells in growth medium) by Olympus (CK 40, Japan) with camera attachment.

After 144 h and 240 h incubation, exposure of cells to polymer was stopped by discarding the growth medium. The numbers of cells survived determined by using MTT assay which measures reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product by using the calibration curve (Appendix D). This assay estimate cell viability and proliferation as follows. After discarding the exposure medium, 0.5 mg/mL of MTT (in Dulbecco's modified PBS) were added to each well and incubated at $37^{o}C$ under 5% CO_2 atmosphere for 4 h. After that, 100 µL of dimethyl sulphoxide (DMSO) was added to each well to dissolve the formazan salts. MCF-7 cells were cultured with microspheres (samples MSA-MSD) and with free drug (sample F0) and for 6 and 10 days. For coculture experiments, MCF-7 and MDA-MB were routinely cultivated in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 mg/mL) at $37^{0}C$, and 5% CO_2 in humidified atmosphere. $1x10^3$ cells were seeded into each well of a 96-well plate and incubated for 24 h at $37^{o}C$. Samples (MSA, MSB, MSC, MSD) in each eppendorf tube were diluted with 1 mL cell culture medium. Then, into the cell cultures 100 µL of samples were added. After, 144 hours incubation period, cells were photographed by a microphotographer. MCF-7 and MDA-MB cells both were cocultured with the microsphere samples (A-D) and with free drug as control for 6 days. Photographs of these cultured and cocultured samples were taken and then cell absorbance of all samples was measured at 570 nm by VersaMax, molecular device, USA.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Optimization and Characterization of Microspheres

In the preparation of microspheres, 2 mL of aqueous GA was used as crosslinker in different concentrations which are 1.25% (v/v), 2.5% (v/v) and 5.0% (v/v). It can be estimated that the structure of microspheres will be lightly crosslinked if GA concentration is low. However, as the concentration of GA increases, microspheres will be harder because of highly crosslinking structure and degradation time of microspheres will be longer. In a controlled release system, therefore it is expected that highly crosslinked microspheres would release their content slowly for extended periods. It was observed that, when the crosslinker concentration was increased, the microspheres became more proper spherical shapes. The microspheres prepared with 5.0% GA solution resulted better shaped microspheres. Also the size of these microspheres was more uniform than the ones prepared with 1.25% GA or 2.5% GA addition (Figure 3.1). Because of these reasons, 5.0% GA concentration was chosen and further experiments were carried with these samples. SEM images of chitosan microspheres prepared with 1.25%, 2.5% and 5.0% GA which are named as CH-1.25, CH-2.5 and CH-5, respectively are given in Figure 3.1.

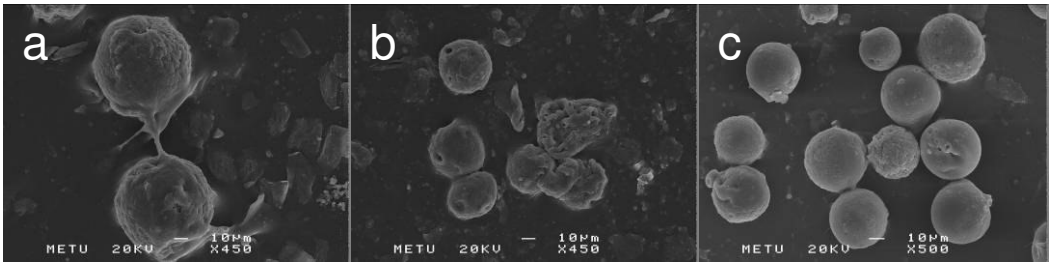


Figure 3.1 SEM results of chitosan microspheres prepared with different amounts of GA (a) CH-1.25, (b) CH-2.5 and (c) CH-5.0

Particle size of of CH-1.25, CH-2.5 and CH-5.0 were measured by Mastersizer particle size analizer distrubiting the particles in water and avarage particle sizes were found 136 µm, 97 µm and 90 µm, respectively (Figure 3.2) (Appendix C).

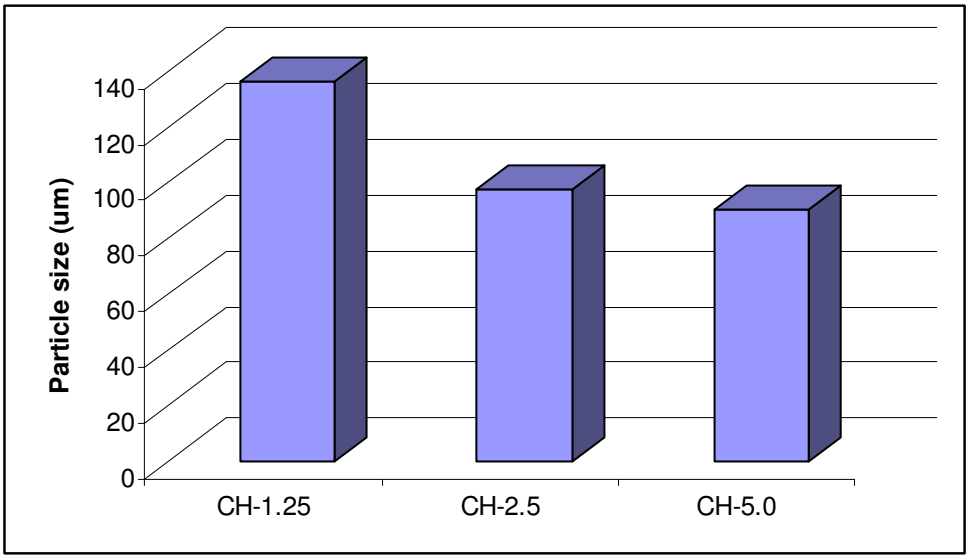


Figure 3.2 Particle size of CH microspheres

After this optimizing of GA concentrations, CH-PVP semi-IPN microspheres were prepared by emulsification method with addition of 2 mL of 5% (v/v) aqueous GA solution. SEM micrographs of CH-PVP semi-IPN microspheres prepared with different amounts of CH and PVP are given in Figure 3.3 (Appendix B). All semi-IPN microspheres seem to have similar structure but are different in size. The surfaces are highly wrinkled for all samples. This might be the result of drying process. After obtaining the microspheres, they were washed with acetone to remove oil and then left to dry. But, presence of highly hydrophilic PVP in the structures caused highly swollen microspheres and these swollen particles became wrinkled after drying. Particle size of semi-IPN microspheres were measured by Mastersizer particle size analizer distrubiting the particles in water and avarage particle sizes were found 170 μm, 159 μm, 153 μm and 90 μm for CH:PVP-1:1, CH:PVP-1:0,5, CH:PVP-1:0,33 and CH respectively (Figure 3.4). Size distribution curves and size of these samples are given in Appendix C.

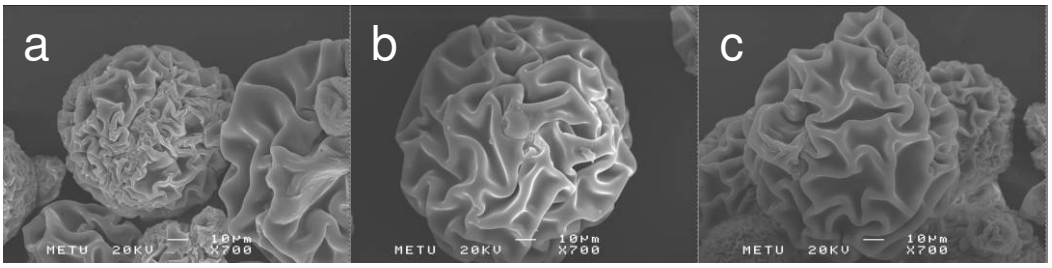


Figure 3.3 SEM pictures of semi-IPN microspheres (a) CH:PVP-1:1, (b) CH:PVP-1:0,5 and (c) CH:PVP-1:0,33

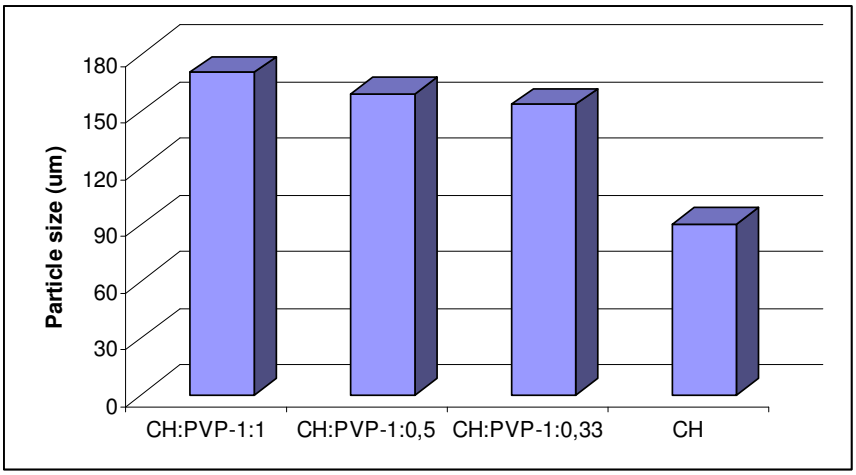


Figure 3.4 Particle sizes of chitosan and semi-IPN microspheres

3.2 Mechanical Properties of the CH-PVP Semi-IPN Films

Chitosan semi-IPN films, prepared at different crosslinking degrees, were analyzed for their tensile properties. The obtained results for all of semi-IPN films are as follows.

The mean ultimate tensile strength (UTS) value of CH:PVP-1:1-1, CH:PVP-1:0,5-1 and CH:PVP-1:0,33-1 films were found as 66.40 MPa, 108.50 MPa and 118.20 MPa, respectively. As can be seen from Table 3.1 as the amount of PVP decreased UTS value was increased. This trend is the same for the other groups prepared with 2.5% and 5.0% GA solutions. This is expected because chitosan has quite high tensile strength because of its highly crystalline structure. Addition of PVP disturbed crystallinity and thus cause a decrease in UTS values. Normally as the crosslinking increases UTS value is expected to be increased. Our results showed that as the crosslinking increased UTS value was unexpectedly decreased. This might be again explained by the bulk formations of crosslinkings, create pre-stressed internal areas probably caused defficiency in crystalline structure of the chitosan structure.

Mean Elastic modulus (E) values of the Chitosan-PVP films with different crosslinking and different content of PVP demonstrated that, as the content of PVP and concentration of crosslinker increases modulus of elasticity changes. But we could not find any relation. This might be again due to bulk formation of PVP or GA in the structure which also leads destruction of the structure.

As can be seen from the Table 3.1, starin at break (SAB) values first increased from 12-15% to 21-28%, but when PVP content decreased further, SAB values dropped to 16-10%. The reason again might be the fromation of bulky regions in the structure due to cross-linking molecules which did not make bond in the matrix.

Table 3.1 Mechanical test values of CH-PVP semi-IPN films

Sample	UTS (MPa)	Modulus (GPa)	SAB
CH:PVP-1:1-1	66.40±6.70	0.912±0.1	12.59 ± 4.6
CH:PVP-1:1-2.5	63.90±9.20	1.187 ±0.128	13.61 ± 2.09
CH:PVP-1:1-5	38.30±1.90	0.637 ±0.104	15.39 ± 1.77
CH:PVP-1:0,5-1	108.50±3.60	1.125±0.059	21.89 ± 4.80
CH:PVP-1:0,5-2.5	65.10±2.70	1.222 ±0.153	23.58 ± 6.20
CH:PVP-1:0,5-5	31.62±7.70	0.839 ±0.165	28.35 ± 4.00
CH:PVP-1:0,33-1	118.20±7.60	1.513±0.115	16.10 ± 0.84
CH:PVP-1:0,33-2.5	70.30±4.40	0.987 ±0.129	19.54 ± 1.82
CH:PVP-1:0,33-5	44.18±2.61	1.055 ±0.250	10.73 ± 3.00

3.3 ATR Characterization

ATR analysis of the films was performed to characterize the functional groups of chitosan and PVP structures by Perkin Elmer Spectrum BX ATR-FTIR system. 32

scans were collected between 4400 cm^{-1} and 600 cm^{-1}. Analysis of the spectra (Figure 3.5) of CH:PVP-1:1-5, CH:PVP-1:0,5-5, CH:PVP-1:0,33-5 blends indicates that interaction exists between these to polymers, probably between chitosan hydroxyl and PVP carboxyl groups (Figure 3.6).

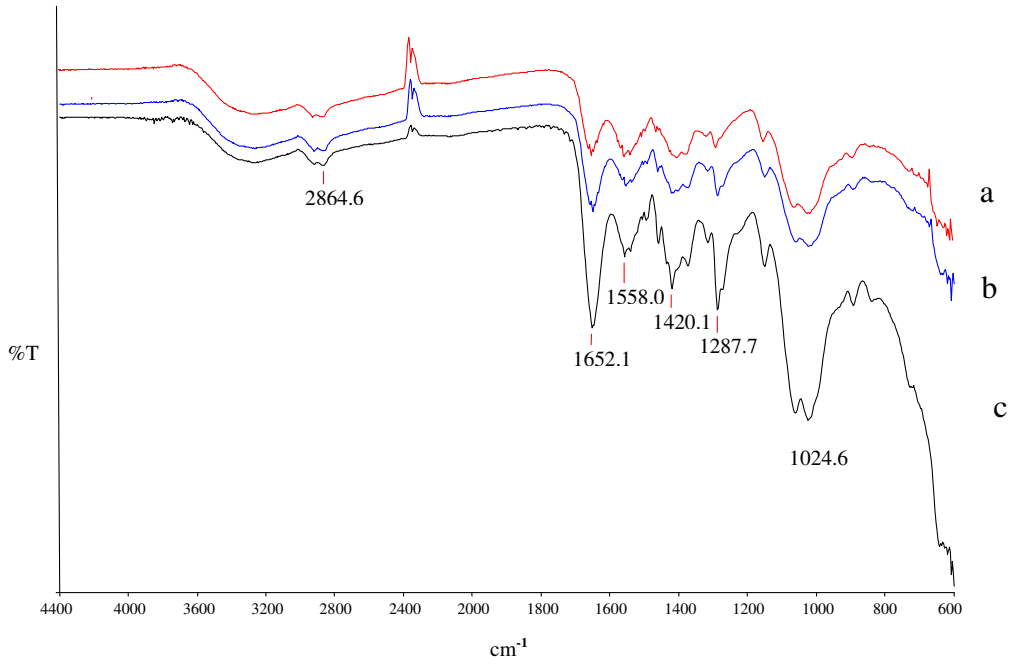


Figure 3.5 ATR spectra of films (a) CH:PVP-1:1-5, (b) CH:PVP-1:0,5-5 and (c) CH:PVP-1:0,33-5

OH absorption for chitosan shifts downwards in frequency from about 3435 to around 3272 cm^{-1}. As can be seen from the spectra the peak at 1652.13 cm^{-1} belongs to carbonyl group (-C=O stretching in amide) in polyvinylpyrrolidone [67]. Normally this peak should be observed at 1688 cm^{-1} but the peak was shifted downward to 1652.13 cm^{-1} because of the interaction between chitosan and PVP.

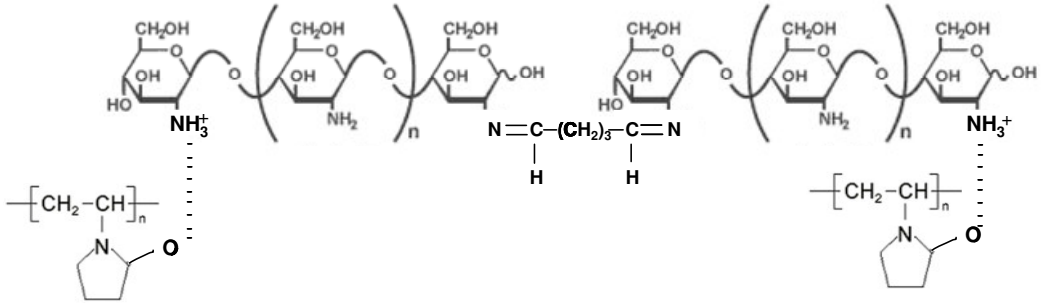


Figure 3.6 Interaction between chitosan and PVP

Both effects indicate hydrogen bonding interactions [68]. It was also observed that the peak intensity increased which is expected because the amount of PVP was increased. The peak around 2700 cm^{-1} is the characteristic peak of NCO in PVP structure but it is shifted to 2864.6 cm^{-1} because of the interaction between chitosan and PVP [78]. The broad peak around 3200 cm^{-1} belongs to OH groups in chitosan. Primary amine groups appear, typically as two peaks in the 3500–3200 cm^{-1} region [79]. But these peaks were not observed in the spectra. This might be caused by crosslinking of chitosan (Figure 3.7).

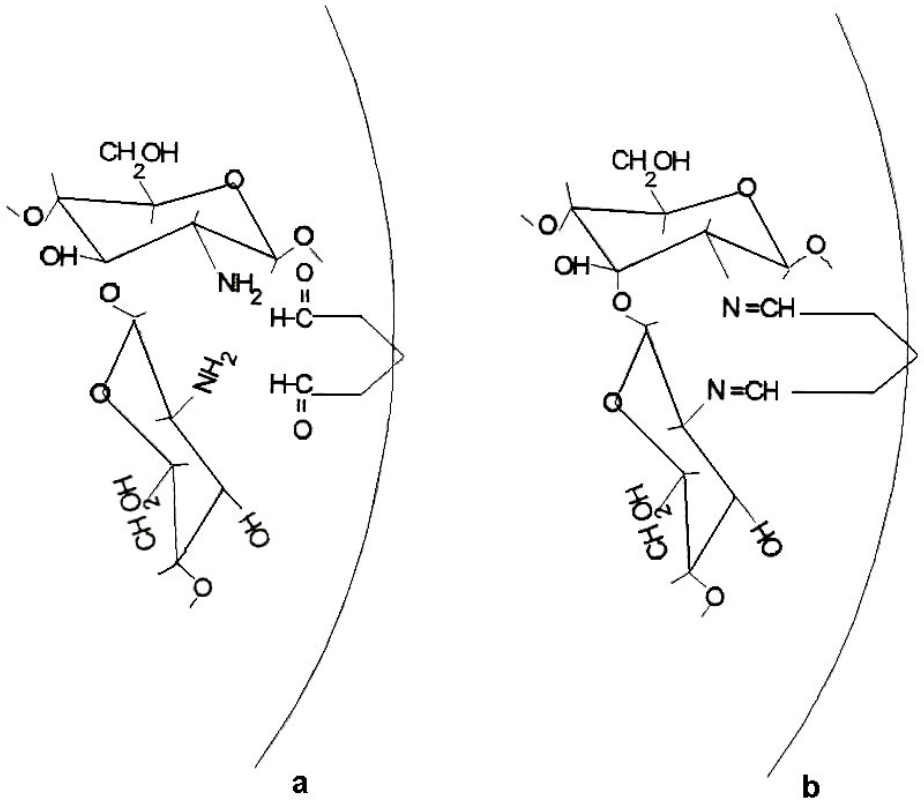


Figure 3.7 Crosslinking reaction of GA with chitosan (a) before crosslinking (b) after crosslinking

3.4 Contact Angle Measurements

Surface hydrophilicity of the prepared films were detected by measuring water contact angles. For statistical approach contact angles of five drops of the water were measured. Unsymmetrical drop values were not included. Contact angles were measured by goniometer and the results are given in Table 3.2. The contact angle values decreased from 97^{o} to 53.3^{o} with increasing concentration of glutaraldehyde for pure chitosan films. But for CH-PVP semi-IPN films, if there is no GA; contact angles achived a continuous decrease from 97.93^{o} to 33.40^{o} with addition of highly hydrophilic PVP, as expected. But 1% GA addition caused

very hydrophobic structures, most probably making covalent bonds with the functional grups of the CH films.

Table 3.2 Contact angle values of films

	No GA	**1.0% GA**	**2.5% GA**	**5.0% GA**
CH	97.93±8.45	71.87±0.89	72.87±0.86	53.57±2.07
CH:PVP-1:0,33	59.83±7.24	104.75±4.83	66.33±0.66	55.12±3.56
CH:PVP-1:0,5	62.79±9.52	94.65±4.23	66.16±1.74	59.18±3.86
CH:PVP-1:1	33.40±1.21	91.12±1.05	60.21±1.27	58.54±3.29

When we consider the blends, introduction of the crosslinker increased the contact angle up to a point (1.0% GA). Then as the concentration of crosslinker increased the contact angle decreased (Figure 3.8). This might be caused by the shrinking of the structure leading hydrophilic moities outside of the film [80]. From another point of view, as the amount of PVP increased, contact angle decreased. This trend is expected because PVP is a hydrophilic polymer.

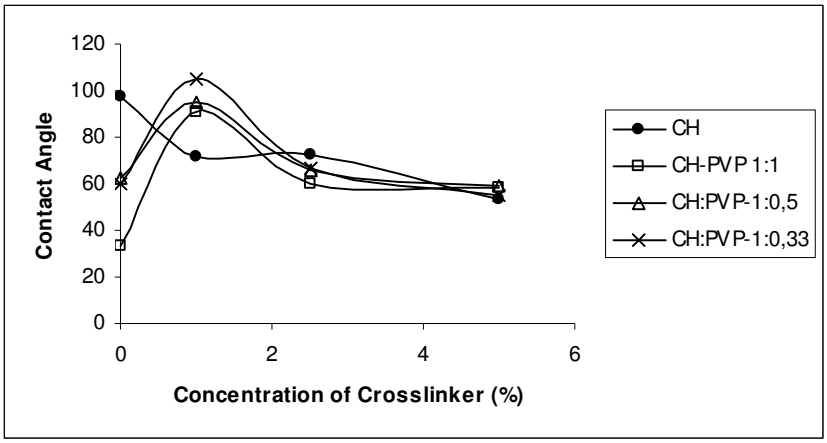


Figure 3.8 Contact angle change with respect to GA concentration for films

3.5 In Vitro Release Studies

5-Fluorouracil (5-FU) showed a maximum absorption at 266 nm in UV spectrum. This wavelength was used for the preparation of calibration curve (Given in Appendix A) and in the detection of released amount of 5-FU from chitosan-polyvinylpyrrolidone microspheres prepared with 5% (v/v) GA addition. Release behaviour of 5-FU from the prepared microspheres is given in Figure 3.9. Entrapment efficiencies of the samples were calculated and it was seen that for semi-IPN microspheres entrapment efficiency increased with increasing amount of PVP in the structure. Entrapment efficiencies were calculated as 12.7%, 23.4% 33.8% and 42.2% for CH, CH:PVP-1:0,33, CH:PVP-1:0,5 and CH:PVP-1:1, respectively (Table 3.3).

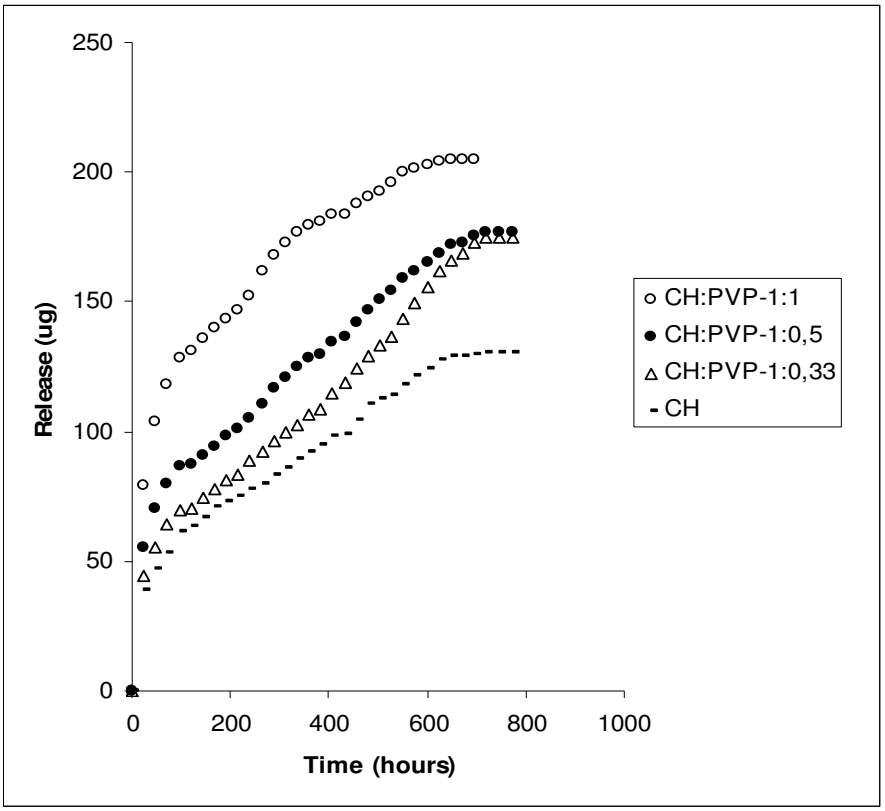


Figure 3.9 Release of 5-fluorouracil from microspheres

Table 3.3 Entrapment values

Sample	Maximum release	Entrapped amount	Entrapment efficiency
CH	130,354 µg	127,1 µg	12.7 %
CH:PVP-1:0,33	174,780 µg	233,3 µg	23.4 %
CH:PVP-1:0,5	176,590 µg	337,3 µg	33.8 %
CH:PVP-1:1	204,795 µg	421,9 µg	42.2 %

The aim of the study was to obtain semi-IPN structures by using crosslinked chitosan and soluble PVP so that PVP dissolve and diffuses out from the matrix and creates channels in which the drug would also diffuse out.

So that; depending on the amount of PVP in the matrix, it would become possible to control the release of the drug. To examine if there are any pores, freeze-dried SEM pictures of CH and CH:PVP-1:1 microspheres were taken (Figure 3.10).

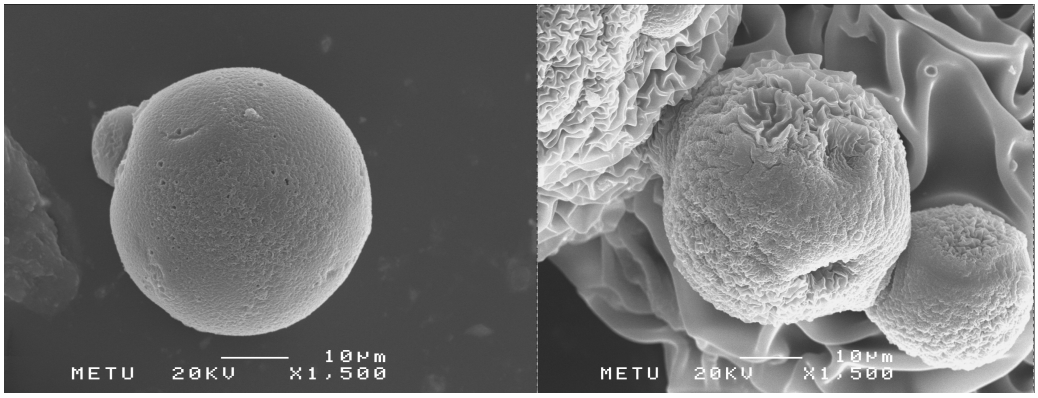


Figure 3.10 Freeze-dried microspheres (a) chitosan (b) CH:PVP-1:1

It was observed that, as the amount of PVP increased the release was increased as expected. Because the release of PVP will cause tortuosity and will change drug release. As a result, drug release is expected to be increased. In addition, initial (first day) and late release rates were studied and it was shown that as the amount of PVP increased the initial rates increased which is also expected because of increase in porosity. Initial and late rates of CH, CH-PVP 1:0,33, 1:0,5 and 1:1 microspheres are shown in Figures 3.11 and 3.12, and the release rate values are given in Table 3.4.

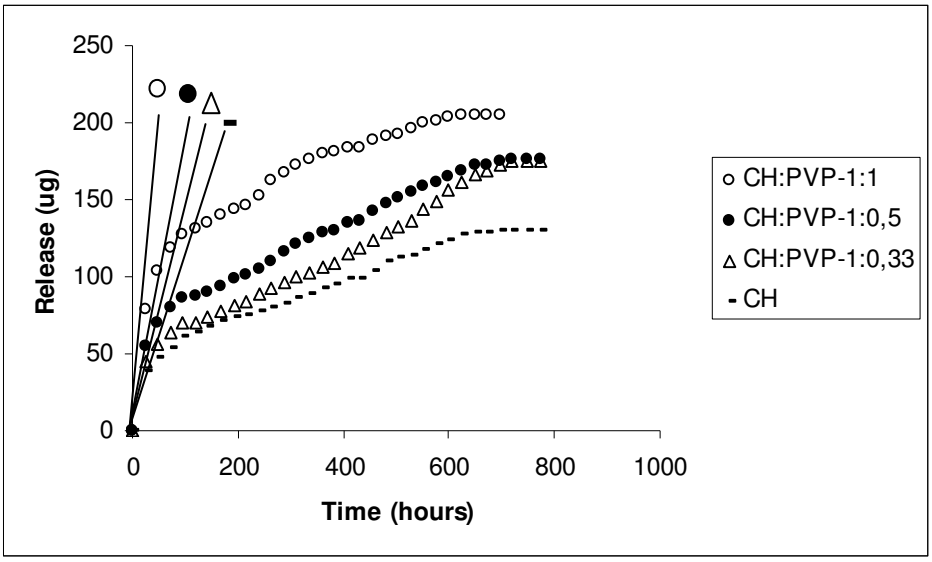


Figure 3.11 Initial release rate profiles of microspheres

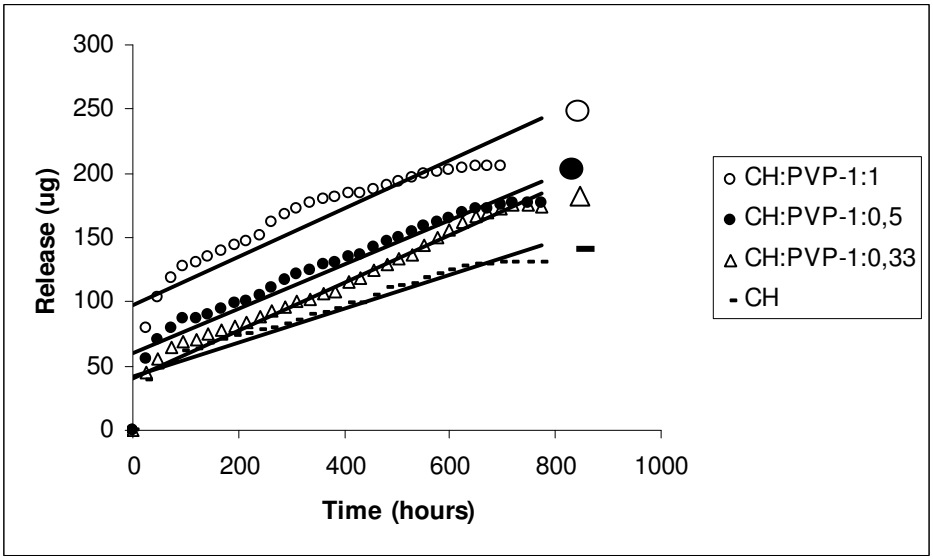


Figure 3.12 Late release rate profiles of microspheres

Late release rates (last 30 days) for CH, CH-PVP 1:0,33, 1:0,5 and 1:1 are 0,10 $\mu g.h^{-1}$ 0,18 $\mu g.h^{-1}$, 0,16 $\mu g.h^{-1}$ and 0,16 $\mu g.h^{-1}$ respectively. When the initial release rates were compared with late release rates (Table 3.4), it can be seen that initial release rates are faster and late release rates are slower. This might be due to release of drug from the surface during the first day (burst effect) and the rest of drug, which is located inner core of the microspheres, would be released in a longer time with a slower rate. After 24 hours, amount of released drug from CH, CH:PVP-1:0,33, CH:PVP-1:0,5 and CH:PVP-1:1 samples were 38,8 µg, 44,57 µg, 55,1 µg and 78,96 µg, respectively. If the maximum amount of released drug is considered as the equilibrium amoung the last data percent release values after 24 hours are 29,8%, 25,5%, 31,2% and 38,5% for CH, CH:PVP-1:0,33, CH:PVP-1:0,5 and CH:PVP-1:1 samples, respectively. The time required for 50% release of drug is 144 hrs, 240 hrs, 120 hrs and 48 hrs for CH, CH:PVP-1:0,33, CH:PVP-1:0,5 and CH:PVP-1:1, respectively.

Table 3.4 Composition and release rates of microspheres

Sample Name	Composition (CH/PVP) (w/w)	Release Rates ($\mu g.h^{-1}$)	
		Initial	Late
CH	-	1,62	0,10
CH:PVP-1:0,33	0.3/0.1	1,85	0,18
CH:PVP-1:0,5	0.3/0.15	2,30	0,16
CH:PVP-1:1	0.3/0.3	3,29	0,16

3.5.1 Release Kinetics

In order to understand the type of drug release kinetics from chitosan microspheres the data were fitted into the standart release equations (zeroth order, first order, Higuchi and Krosmeyer equations). Best fit was observed with Higuchi model ($M_t/M_0=Kt^{1/2}$) for CH microspheres, CH:PVP-1:0,5 and CH:PVP-1:0,33 semi-IPN microsphere samples because percent release is directly proportional to square root of time (Figure 3.13 C). Also while considering the highest correlation coefficient value (R^2), the release data seem to fit the Higuchi model better. According R^2 values, CH:PVP-1:1 microspheres fitted to Krosmeyer model (Table 3.5).

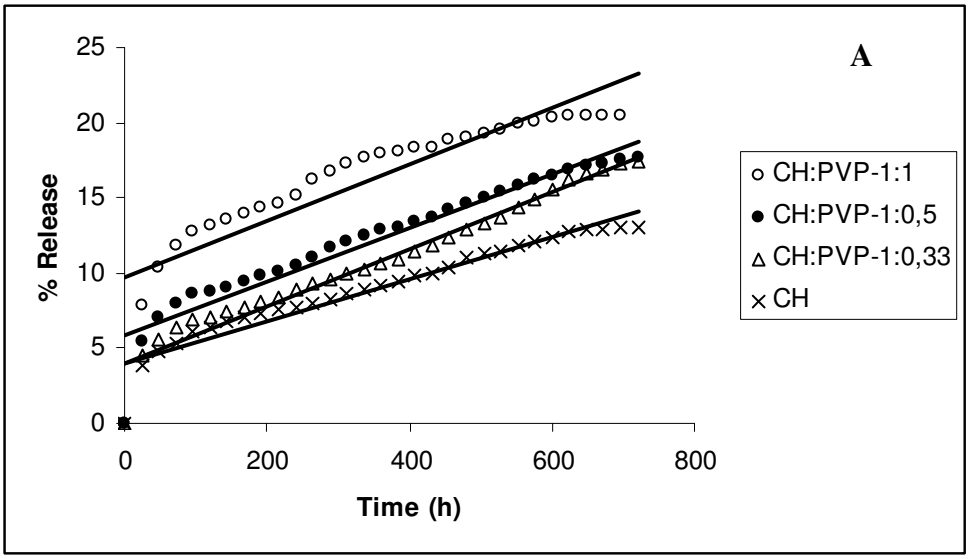


Figure 3.13 Plot of kinetic data in accordance with release models (A) zeroth, (B) first order, (C) Higuchi and (D) Krossmeyer models

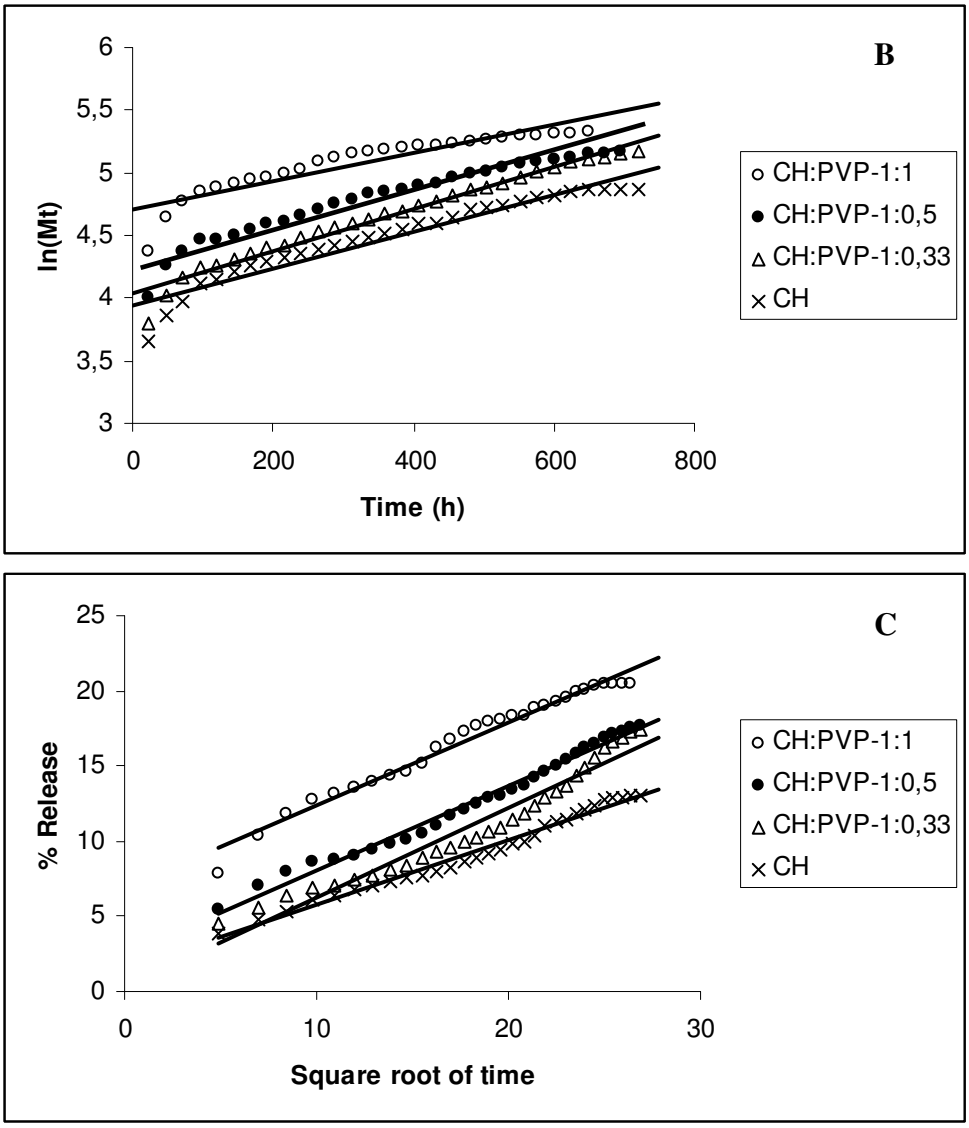


Figure 3.13 cont'd

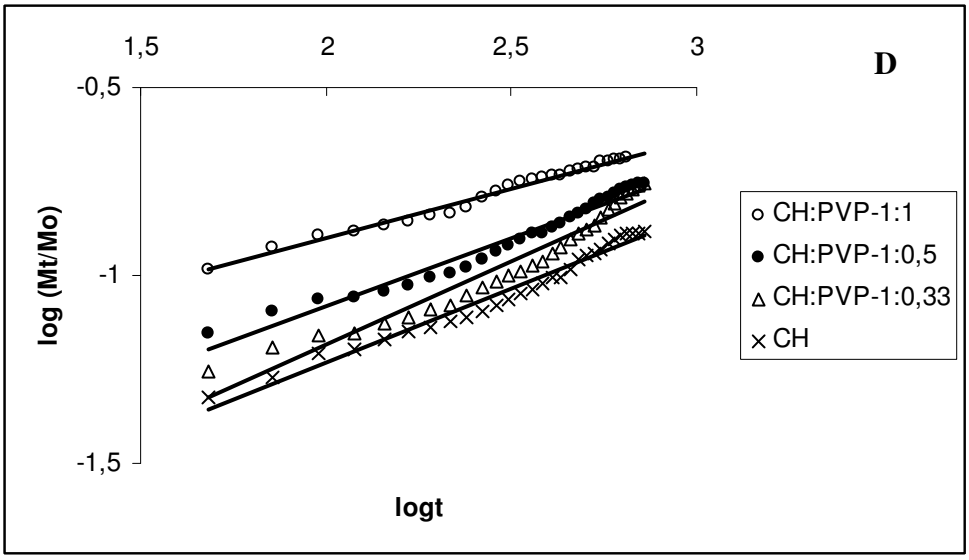


Figure 3.13 cont'd

Table 3.5 Correlation coefficients of CH-PVP semi-IPN systems

Samples	Correlation Coefficient (R^2)			
	0th Order	1st Order	Higuchi	Krosmeyer
CH	0,9268	0,8367	**0,9852**	0,9739
CH:PVP-1:0,33	0,9268	0,9303	**0,9568**	0,9383
CH:PVP-1:0,5	0,9154	0,9695	**0,9879**	0,9649
CH:PVP-1:1	0,7719	0,9370	0,9782	**0,9888**

3.6 Degradation Studies

In order to study degradation kinetics of microspheres, 10 mg of microspheres were incubated in lysozyme solution (30 mg lysozyme in 10mL PBS). Because chitosan is degraded by lysozyme which commonly exists in various human body fluids and tissues [81]. At the beginning, in every 24 hours small amount of microspheres were added and examined by stereo microscopy.

Photographs of these microspheres are given in Figure 3.14. From stereo microscopy photographs, it was not clear whether the microspheres degraded or not. But it was observed that there are some aggregations among them. In order to examine the change in the shapes of microspheres by degradation, SEM pictures were taken and images are given in Figure 3.15.

A) After 2 days

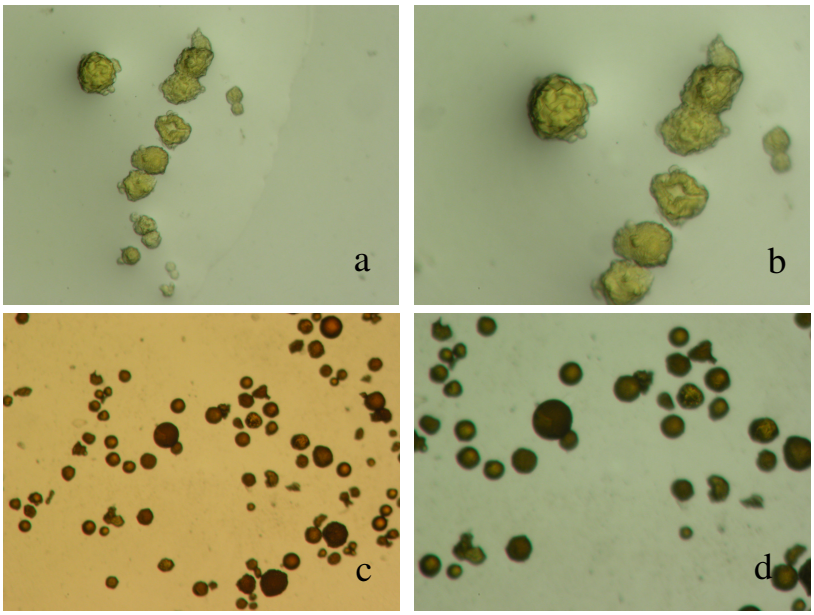


Figure 3.14 Stereo microscopy pictures of microspheres (A) incubated in lysozyme solution for 2 days (a) CH (3x7 magnification) (b) CH (3x11.25 magnification) (c) CH:PVP-1:1 (3x7 magnification) (d) CH:PVP-1:1 (3x11.25 magnification); (B) incubated in lysozyme solution for 17 days (e) CH (3x7 magnification) (f) CH (3x11.25 magnification) (g) CH:PVP-1:1 (3x7 magnification) and (h) CH:PVP-1:1 (3x11.25 magnification)

B) After 17 days

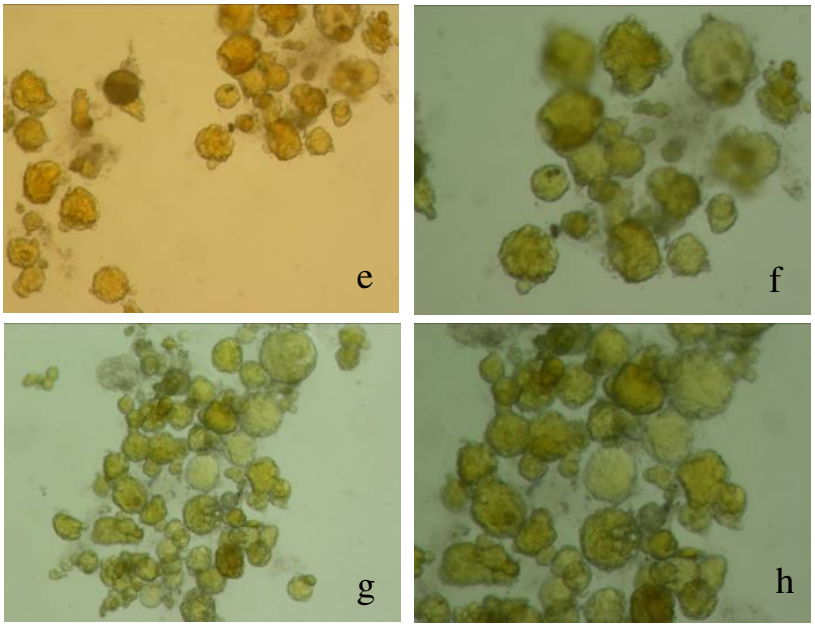


Figure 3.14 cont'd

As can be seen from Figure 3.15, the shapes of microspheres were disturbed. Microspheres in lysozyme solution were slowly degraded. It is expected that lysozyme would attack to chitosan and break its bonds causing degradation. But these reactions were very slow and even after 60 days, the complete degradation was not observed.

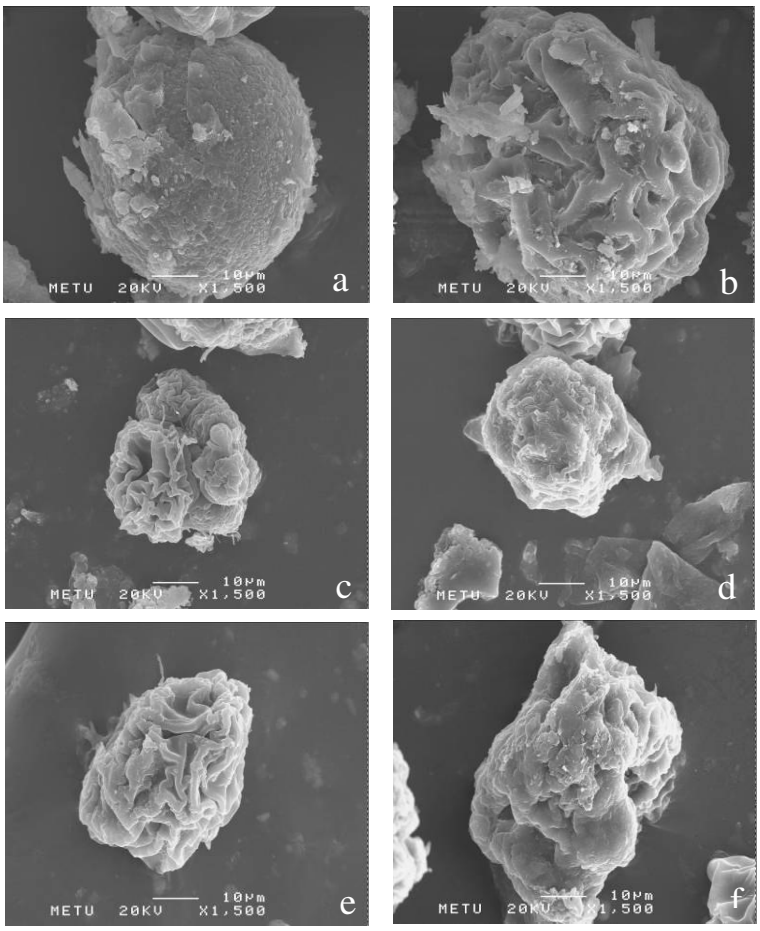


Figure 3.15 SEM images of microspheres (prepared with 5% GA) incubated in lysozyme solution for 60 days (a) CH for 2 days, (b) CH:PVP-1:1 for 2 days, (c) CH for 15 days, (d) CH:PVP-1:1 for 15 days, (e) CH for 60 days and (f) CH:PVP-1:1

3.7 Conjugation of microspheres

Prepared CH:PVP-1:1 microspheres were conjugated with immunoglobulin G antibody. Carboxylic acid groups at the end of the attachment site react with EDC as shown in Figure 3.16 to form an activated peptide intermediate. Then the activated IgG reacts with amine group of chitosan to form IgG-chitosan conjugate by yielding urea.

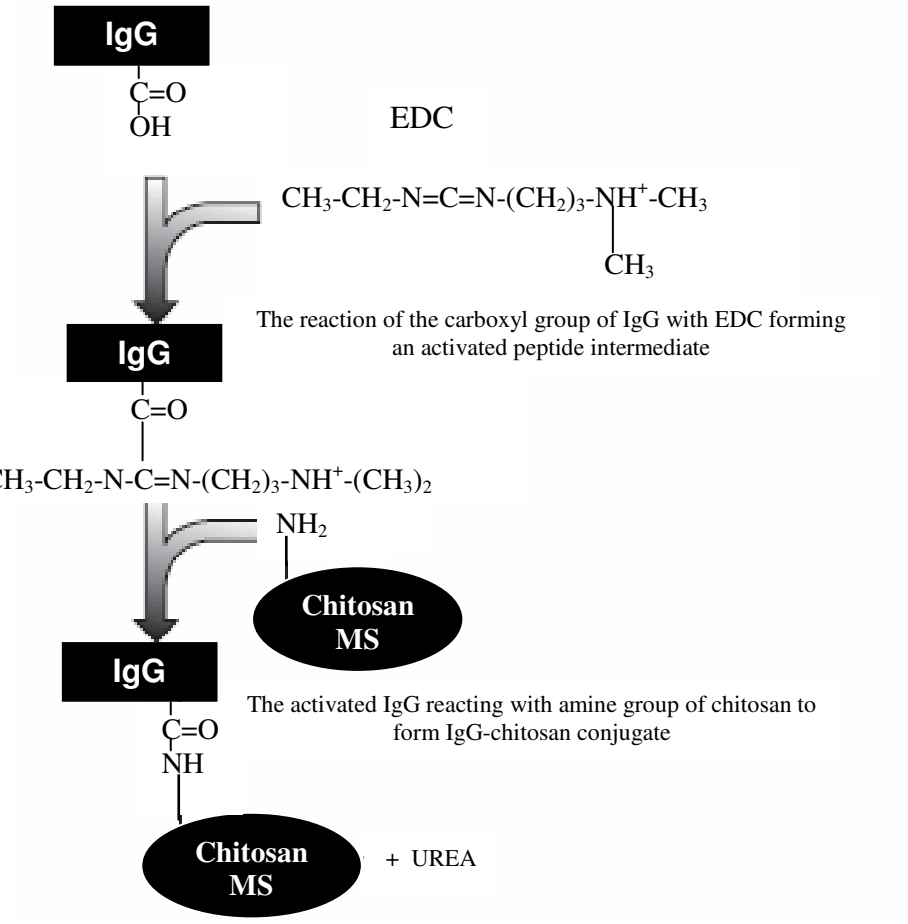


Figure 3.16 Conjugation of microspheres

To investigate microspheres conjugated with IgG, confocal microscopy was used. However, it was not easy to distinguish whether IgG moities were conjugated or not, because chitosan, IgG and glutaraldehyde give emission at the same wavelength and both IgG conjugated or not, in both cases microspheres were all the same colour (Figure 3.17). The conjugation could not be detected by confocal microscopy. In order to investigate whether conjugated microspheres would recognize these cells via IgG moities or not, cell culture experiments were achived by using MCF-7 cells. These cells are well characterized estrogen receptor

positive cell line and therefore they are useful in vitro model of breast cancer studies.

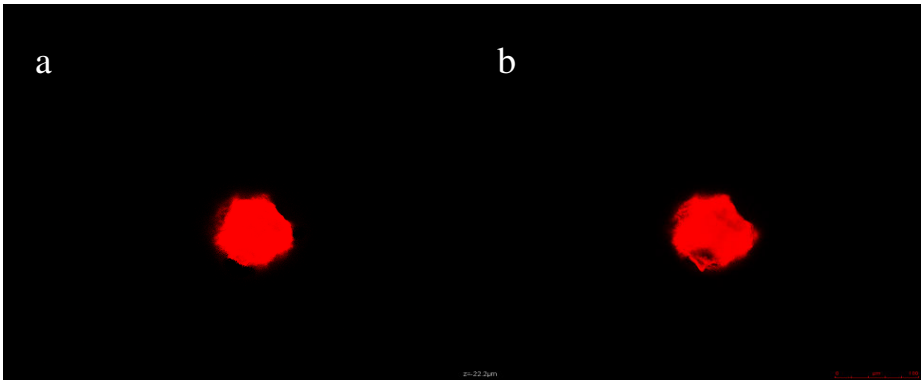


Figure 3.17 Confocal microscopy picture of conjugated and unconjugated microspheres (a) unconjugated and (b) conjugated

3.8 Cell Culture Studies

The pictures of all sets for MCF-7 cell culture are given in Figure 3.18. When we compare the set in which only pure 5-FU drug (1 mL, 50 mg active agent) and with control group, it was obvious that almost all cells were dead (Figure 3.18 b). The same result was obtained for the set of 10 % drug solution (100 μL, 5 mg active agent) (Figure 3.18 c). For the other sets of microspheres (MSA, MSB, MSC, MSD), significant differences were not observed because targeted microspheres are not small enough to be able to interact with the cells separately.

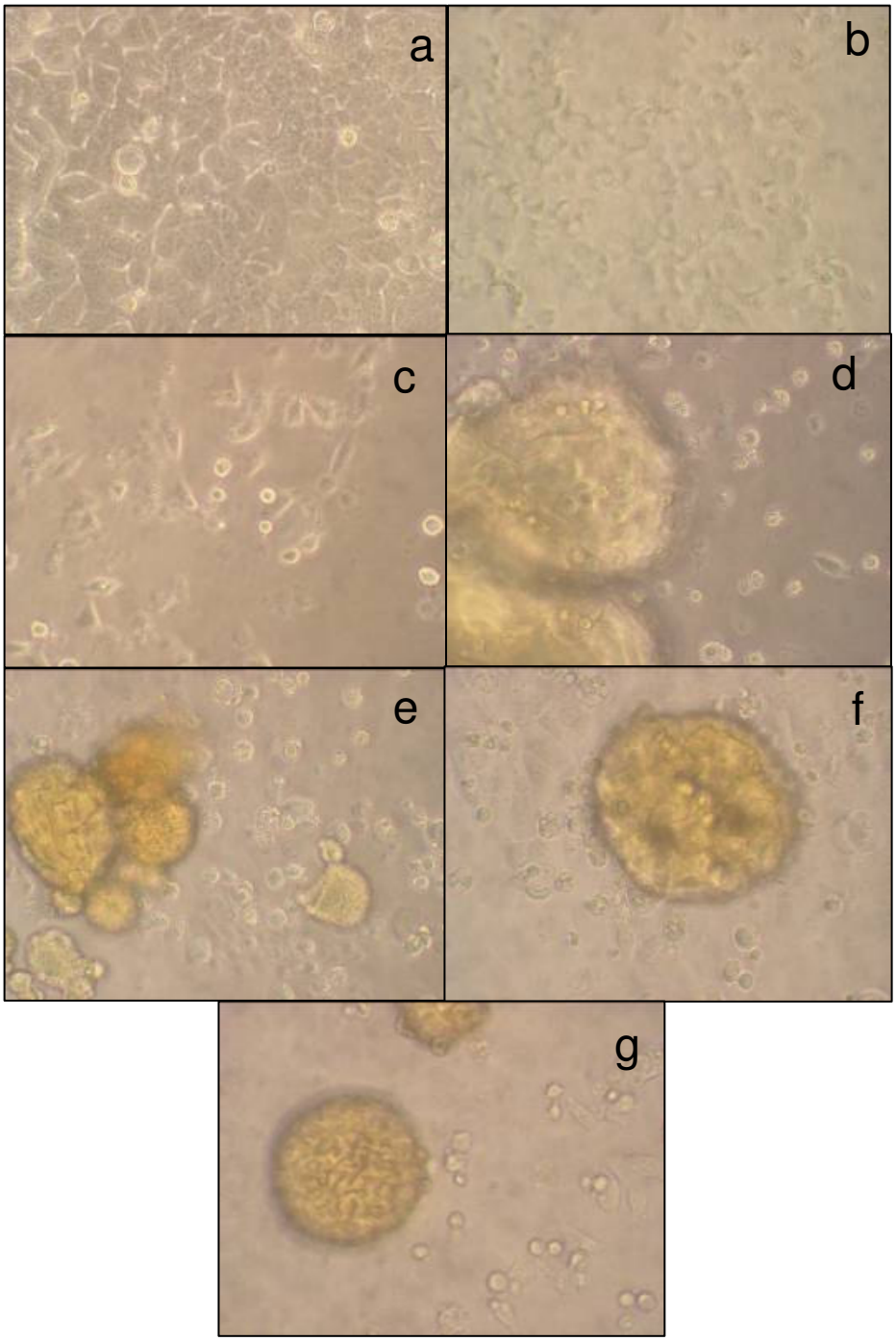


Figure 3.18 Pictures of MCF-7 cell culture after 6 days (a) control (only cells), (b) drug solution (1 mL, 50 mg active agent), (c) 10 % drug solution (5mg active agent), (d) unloaded microsphere (MSA), (e) loaded microsphere (MSB), (f) conjugated loaded microsphere (MSC) and (g) conjugated unloaded microsphere(MSD)

In Figure 3.18 d-e-f-g, MCF-7 cells which are tightly attached to the flask surface, could not be observed. There were no significant differences among the pictures of sample MSA, MSB, MSC and MSD. MCF-7 cells were chosen because these cells are well characterized estrogen receptor positive control cell line and therefore they are useful in vitro model of breast cancer studies. MDA-MB cells do not have those receptors. Therefore, it was proposed that, conjugated microspheres would be recognized by the cells which have receptors if a coculture medium is prepared. MCF-7 and MDA-MB cell coculture is shown in Figure 3.19. MCF-7 cells were epithelial cells tightly attached to the flask surface and MDA-MB cells are round shaped and loosely attached to the flask surface.

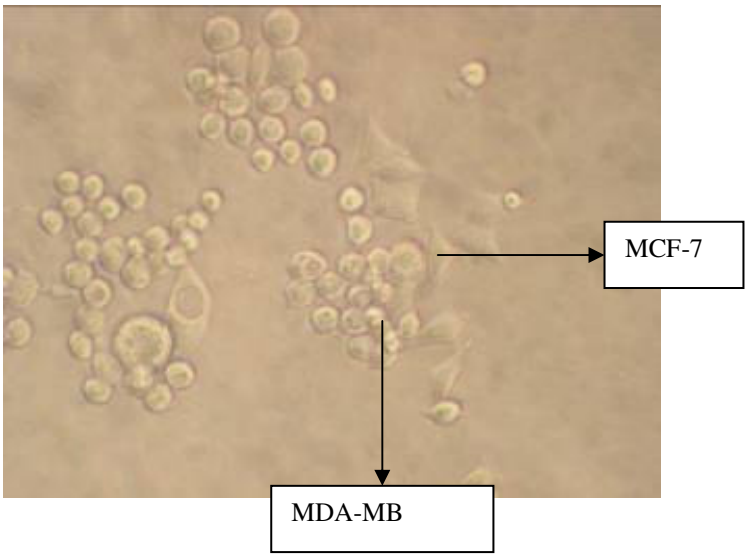


Figure 3.19 Picture of MCF-7 and MDA-MB cell coculture

The cell numbers were detected by MTT test and cell absorbance was determined. Results are given in Table 3.6, Figure 3.20 and 3.21. Since the effect of IgG binding was not observed, the experiments were carried out with unconjugated microspheres. For the control grup, the absorbance of the cells was 1.23 after 6

days. When 5-FU (50 mg) and diluted 5-FU (5 mg) were added absorbance values dropped to 0.12 and 0.20 after 6 days, respectively. Unloaded CH:PVP-1:1 microspheres (MSA), drug loaded CH:PVP-1:1 microspheres (MSB), conjugated drug loaded CH:PVP-1:1 microspheres (MSC) and conjugated unloaded CH:PVP-1:1 microspheres (MSD) all demonstrated very similar results causing a decrease of 1.0 unit in absorbance. After 10 days, there were no alive cells in the medium when drug and diluted drug was used. But for sample MSA and sample MSB we obtained absorbance values to be 0.483 and 0,459, respectively. Cell numbers were obtained as $2.570x10^3$ and $2.406x10^3$ for A and B, respectively. But after 6 days, these values were $0.487x10^3$ and $0.801x10^3$ for MSA and MSB, respectively. This difference might be caused by changing the feeding medium and also possible existance of PVP in the medium at the begining. Existance of PVP might have prevented mass transport in the medium, which may prevent feeding of the cells causing a decrease in the number of the cells. It was expected that, the sample MSC absorbance value would be the least one since these microspheres are conjugated and drug loaded samples. So the cells were expected to recognize the antibody on the surface of microspheres and would interact and die there. But the values were found to be very close to each other.

Table 3.6 MCF-7 cell number after 6 and 10 days

Sample	Absorbance after 6 days	Cell Number (6 days)	Absorbance after 10 days	Cell Number (10 days)
Control	1.23	$7.665x10^3$	1.24	$7.751x10^3$
Drug solution	0.12	$0.101x10^3$	0.0053	0
10% drug solution	0.20	$0.637x10^3$	0.0087	0
MSA	0.18	$0.487x10^3$	0.48	$2.570x10^3$
MSB	0.22	$0.801x10^3$	0.46	$2.406x10^3$
MSC	0.16	$0.355x10^3$	-	-
MSD	0.16	$0.358x10^3$	-	-

This unexpected results might be caused by crosslinking agent of glutaraldehyde which is known as a toxic substance. Secondly, almost impossible equal addition of microspheres into the cell containing compartments. So that some compartments might have more microspheres than the others, leading different amount of drug. Another possibility is the size of microspheres which were quite large (170-150 μm) and cells might not recognize IgG moities on the surfaces of microspheres effectively. In order to measure the pH of the release medium, 0.02 g microspheres were put in to 20 mL distilled water and left for 6 days. The pH of release medium was measured and obtained as 6.5. pH of distilled water was 6.8. Decrease in pH by 0.3 was not tought to be effective on cell death. To be sure about these results, more experiments should be carried out.

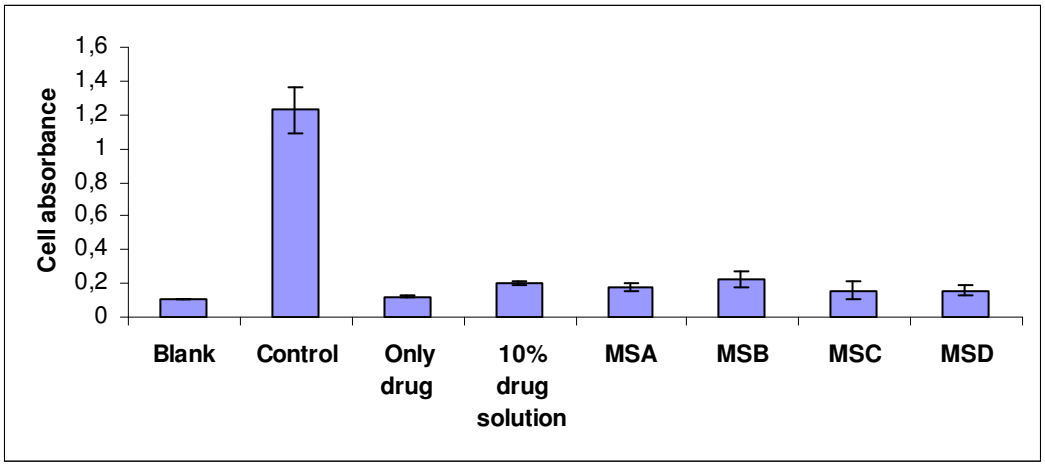


Figure 3.20 Cell absorbance of MCF-7 cell culture after 6 days

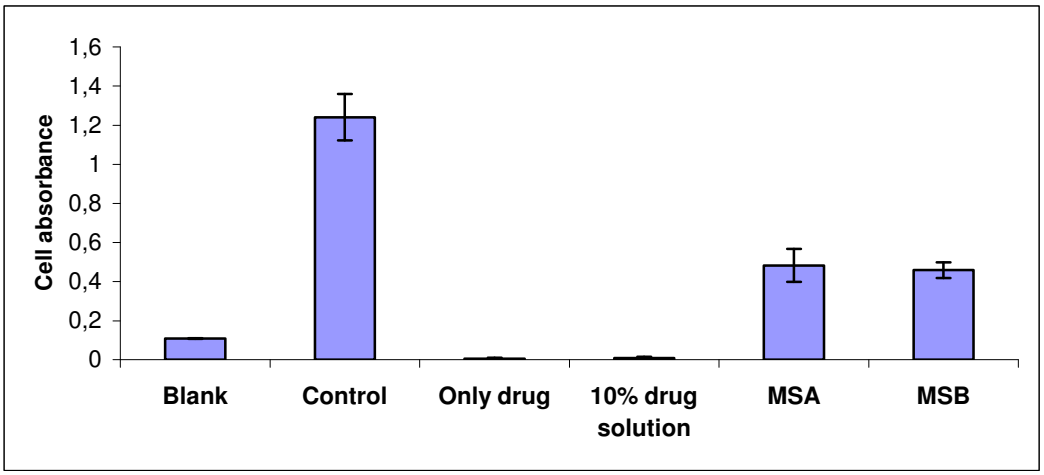


Figure 3.21 Cell absorbance of MCF-7 cell culture after 10 days

After 6 days culturing of MCF-7 and 10 days of coculturing MCF-7/MDA-MB cells with the samples, pictures were taken to observe the differences. As can be seen from Figure 3.22 b, 3.22 c almost all, and from Figure 3.23 b and 3.23 c all cells were dead as expected. For these samples only pure 5-FU solutions (containing 50 mg or 5 mg 5-FU in 100 μL) were used, respectively. For the samples of MSA, MSB, MSC and MSD, MCF-7 cells were dead and MDA-MB cells were alive but with decreased population (Figure 3.22 d-e-f-g). It was not expected that unloaded chitosan microspheres would have a toxic effect and would kill the cells. This might be caused by the release of PVP from the microspheres to the medium. Existance of PVP and its release to the growth medium increases the viscosity of the medium which may prevent the mass-transfer of feeding medium. Moreover it was expected that the drug loaded IgG conjugated microspheres would kill cells more. But the observations were reverse. This might be because of loosing the drug during IgG conjugation step. In this step microspheres were incubated for 24 hours in the PBS solution and according to the results of release experiments most of the drug was released in the first 24 hours (burst effect). Therefore, most probably the drug concentration was lower than expected. So it might not be enough to kill the cells effectively.

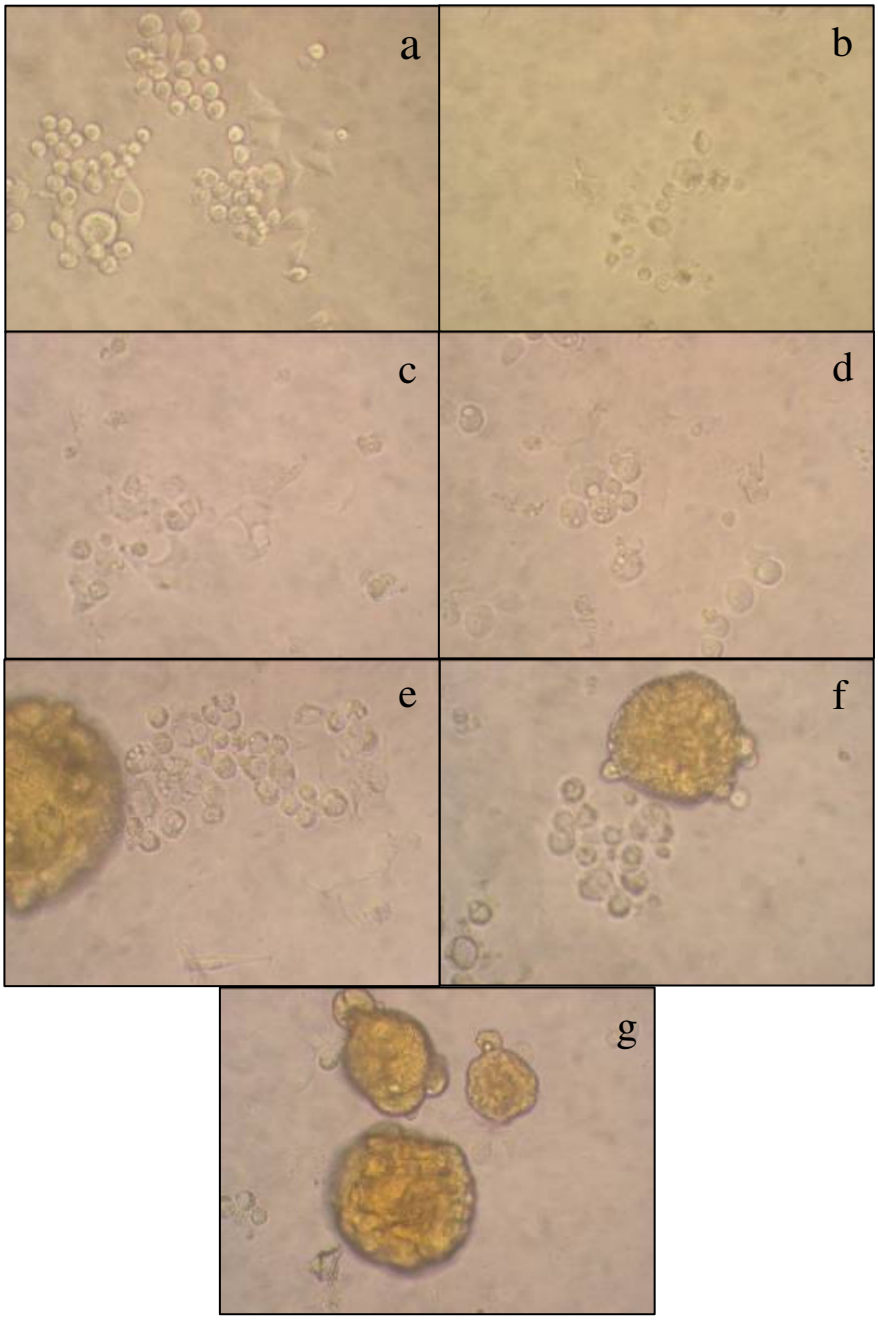


Figure 3.22 Pictures of MCF-7/MDA-MB cell coculture after 6 days (a) control, (b) 5-FU (50 mg), (c) 5-FU (5 mg), (d) CH:PVP-1:1 microspheres, (e) drug loaded CH:PVP-1:1 microspheres, (f) conjugated drug loaded CH:PVP-1:1 microspheres and (g) conjugated unloaded CH:PVP-1:1 microspheres

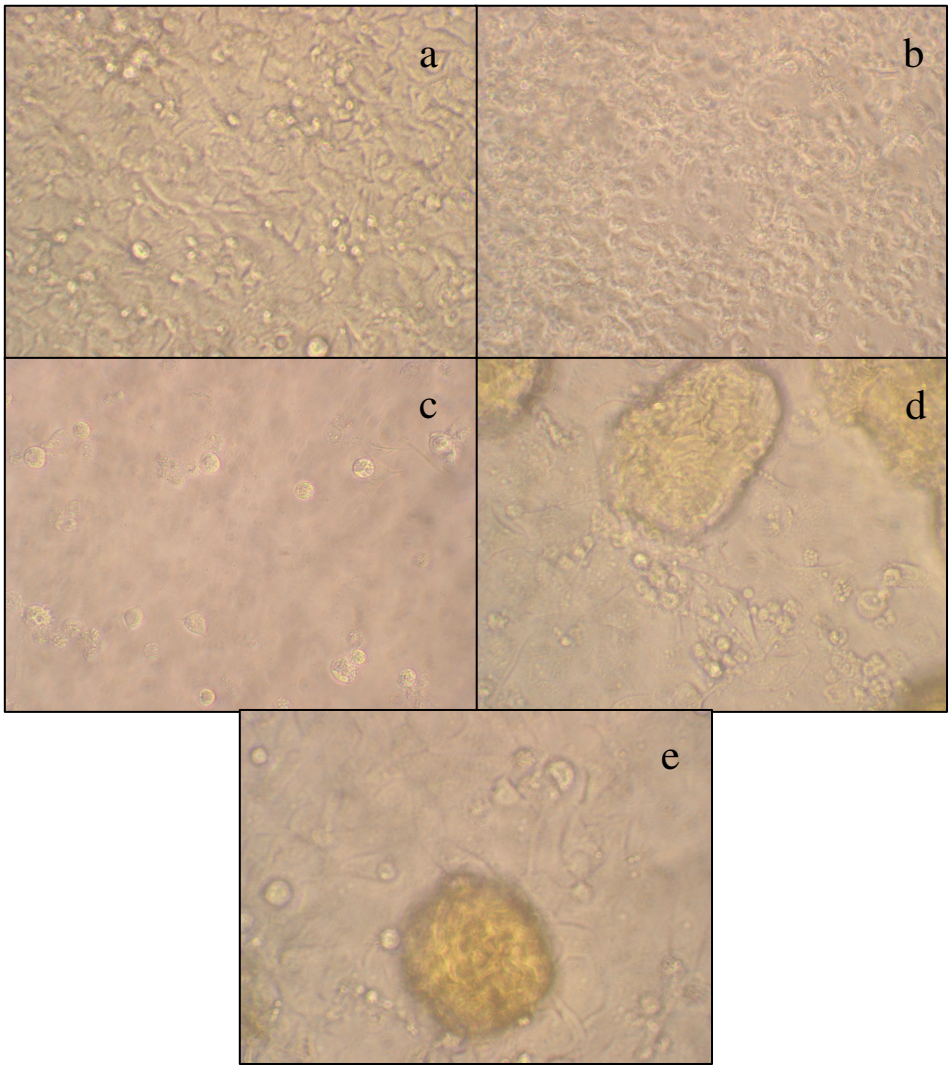


Figure 3.23 Pictures of MCF-7/MDA-MB cell coculture after 10 days (a) control, (b) 5-FU (50 mg), (c) 5-FU (5 mg), (d) CH:PVP-1:1 microspheres, (e) drug loaded CH:PVP-1:1 microspheres

Additionally, cell culture experiments repeated by exposing the cells only to PVP to examine whether there is a relation between cell death and PVP existance in the medium. In this experiment 6 samples of 0,5 mg/mL PVP and 6 samples of 1,0 mg/mL PVP solutions, in feeding medium, were prepared separately and 1 mL of these solutions were added to the growth medium directly. Absorbance results

have shown that cells were still alive that is PVP does not have toxic effect (Figure 3.24).

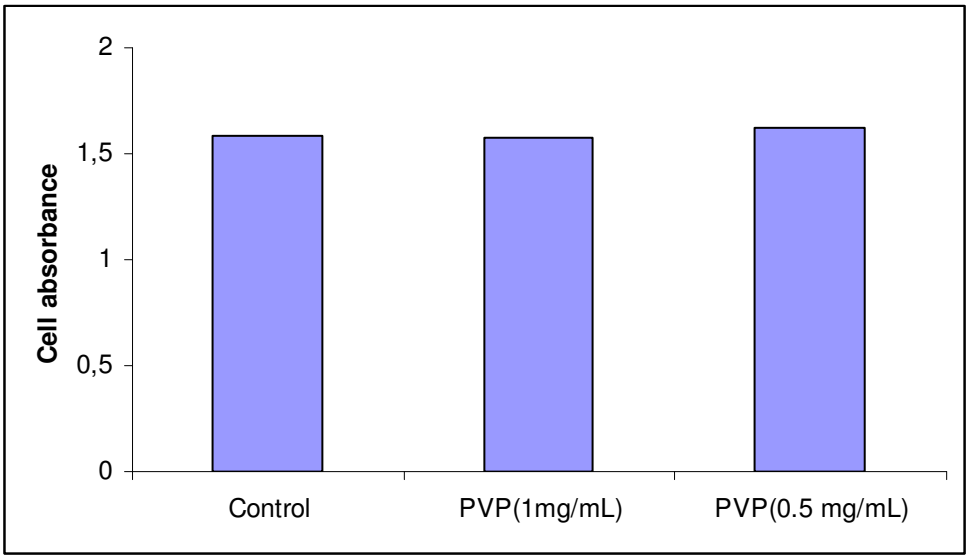


Figure 3.24 Cell absorbance graphic for polymer toxicity experiment

The cell culture tests have shown that chitosan microspheres and CH-PVP semi-IPN microspheres had some toxic effects even they do not contain any 5-FU. Chitosan is known as non toxic and biocompatible polysaccharide and therefore these results are contradictory the literature results. The reason for the toxicity of chitosan might be the existance of high amount of glutaraldahyde or the presence of acetic acid in the microspheres. To be sure about these more experiments are needed.

CHAPTER 4

CONCLUSION

Chemotherapy is one of the major treatments in cancer therapy. However, it is often associated with severe side effects due to the fact that anticancer drugs are primarily cytotoxic agents that not only kill cancer cells but also cause damage to normal cells. In order to decrease or get rid of these severe side effects, chemotherapeutic agents are tried to be targeted to cancer cells in a controlled manner. In this study controlled release of a chemoterapeutic agent, 5-fluorouracil, was studied. For this purpose, chitosan-polyvinylpyrrolidone semi-IPN matrices were prepared in the form of microspheres and films and crosslinked with glutaraldehyde. 5-Fluorouracil was loaded in to the microspheres during the Preparation process. The effect of polyvinylpyrrolidone content on 5-FU release were examined. Then semi-IPN microspheres conjugated with IgG and tried to be targeted to MCF-7 (human breast adenocarcinoma) cells. To examine the specific effect to cancer cells, MCF-7/MDA-MB coculture cell experiments were performed. The results can be summerized as;

- Increasing the concentration of GA caused properly spherical and homogeneously uniform sized microspheres.
- Surfaces of microspheres were wrinkled when PVP was introduced.
- The mean diameters of microspheres was 90 μm when there was no PVP and were decreased from 170 μm to 153 μm as the amount of PVP increased from 25% to 50%.
- Degradation of microspheres was studied for 60 days and it was observed that spherical shape of microspheres changed and the size of microspheres was decreased.

- Initial release rate from microspheres was increased as the amount of PVP increased in microspheres. Values were 1,62 $\mu g.h^{-1}$, 1,85 $\mu g.h^{-1}$, 2,30 $\mu g.h^{-1}$, 3,29 $\mu g.h^{-1}$ and for CH, CH:PVP-1:0,33, CH:PVP-1:0,5 and CH:PVP-1:1 respectively.
- Maximum release and entrapment efficiency were increased as the amount of PVP increased in the structure. Entrapment efficiencies were calculated as 12.7%, 23.4%, 33.8% and 42.2% for CH, CH:PVP-1:0,33, CH:PVP-1:0,5 and CH:PVP-1:1, respectively.
- Release kinetic studies showed that CH and CH:PVP-1:0,5 semi-IPN microspheres fitted into Higuchi model, CH:PVP-1:1 fitted into Krosmeyer model and CH:PVP-1:0,33 fitted into zeroth order release.
- For the control grup cell absorbance was 1.23, when 5-FU (50 mg) and diluted 5-FU (5 mg) were added the absorbance values were dropped to 0.12 and 0.20, respectively after 6 days. Unloaded CH:PVP-1:1 microspheres (MSA), drug loaded CH:PVP-1:1 microspheres (MSB), conjugated drug loaded CH:PVP-1:1 microspheres (MSC) and conjugated unloaded CH:PVP-1:1 microspheres (MSD) all demonstrated very similar results causing a decrease in absorbance about 1.0 unit.
- Cell studies showed that chitosan and CH-PVP semi-IPN microspheres all had some toxic effects on cells
- Cell studies showed that polyvinylpyrrolidone has no toxic effect on cells.

Chitosan-polyvinylpyrrolidone semi-IPN films were prepared by crosslinking with glutaraldehyde and mechanical properties were examined. The results can be summarized as;

- Mechanical test results showed that crosslinked chitosan-polyvinylpyrrolidone semi-IPN films had enough strength to handle them. Tensile strength, elastic modulus and strain at break values were in the range 66.4-118.2 MPa, 0.9-1.5 GPa and 12.6-16.1% for films crosslinked by 1.0% GA; 63.9-70.3 MPa, 0.9-1.2 GPa and 13.6-19.54% for films

crosslinked by 2.5% GA; and 38.3-44.2 MPa, 0.6-1.0 GPa and 10.7-28.4% for the films crosslinked by 5.0% GA, respectively.

- Contact angle values decreased as the amount of PVP increased and no exact relation was observed between crosslinking and contact angle. The values were in the range of 71.8-104.7^{o} for the films crosslinked by 1.0% GA; 60.2-72.9^{o} for the films crosslinked by 2.5% GA and 53.5-59.2^{o} for the films crosslinked by 5.0% GA.

As a result, chitosan and chitpsan-PVP semi-IPN microspheres are promosing devices for the controlled release of 5-Fluorouracil. By changing the ratio of chitosan/PVP, it becomes possible to adjust the release rates of drugs. But the cell culture studies demonstrated that all the microspheres prepared in this study has some toxic effects even in the case when no chemoterapeutic drug (5-FU) was added into the microspheres. Therefore, further optimization of the microsphere chemistry and size as well as in-vivo studies are required to determine the optimum conditions.

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APPENDIX A

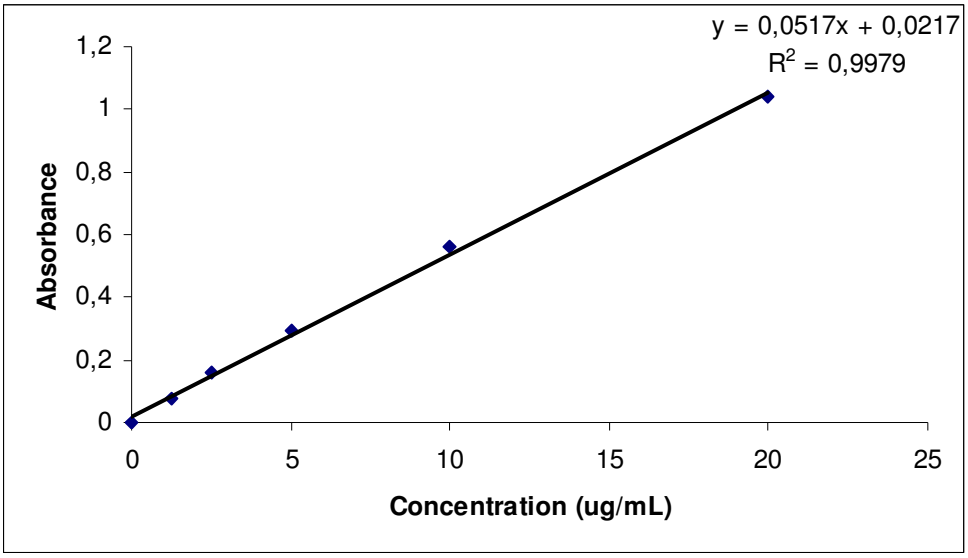

Figure A.1 Calibration curve of 5-Fluorouracil

APPENDIX B

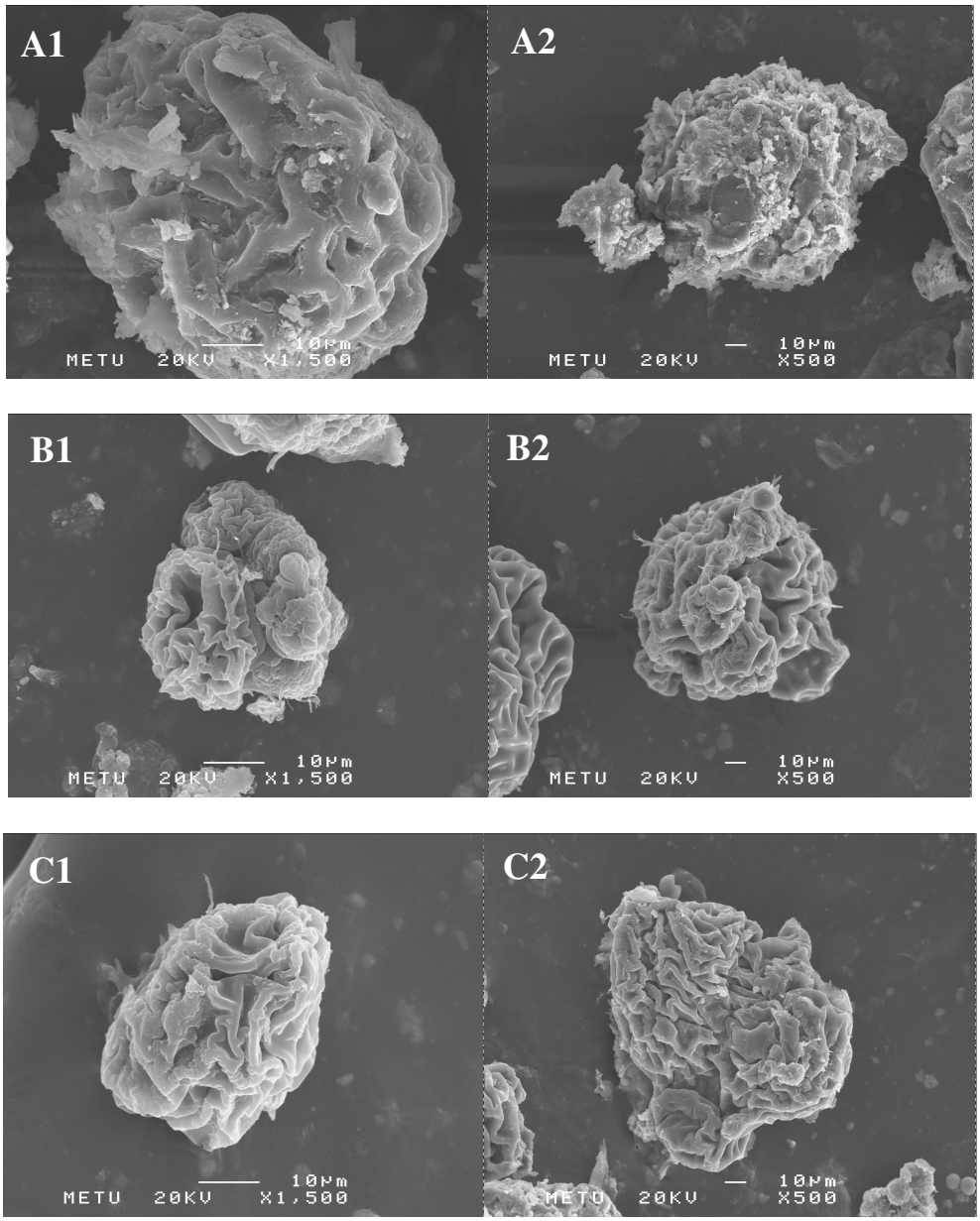

Figure B.1 SEM images of CH microspheres incubated in lysozyme solution for 2 days (A1, x1500; A2, x500), for 15 days (B1, x1500; B2, x500) and for 60 days (C1, x1500; C2, x500)

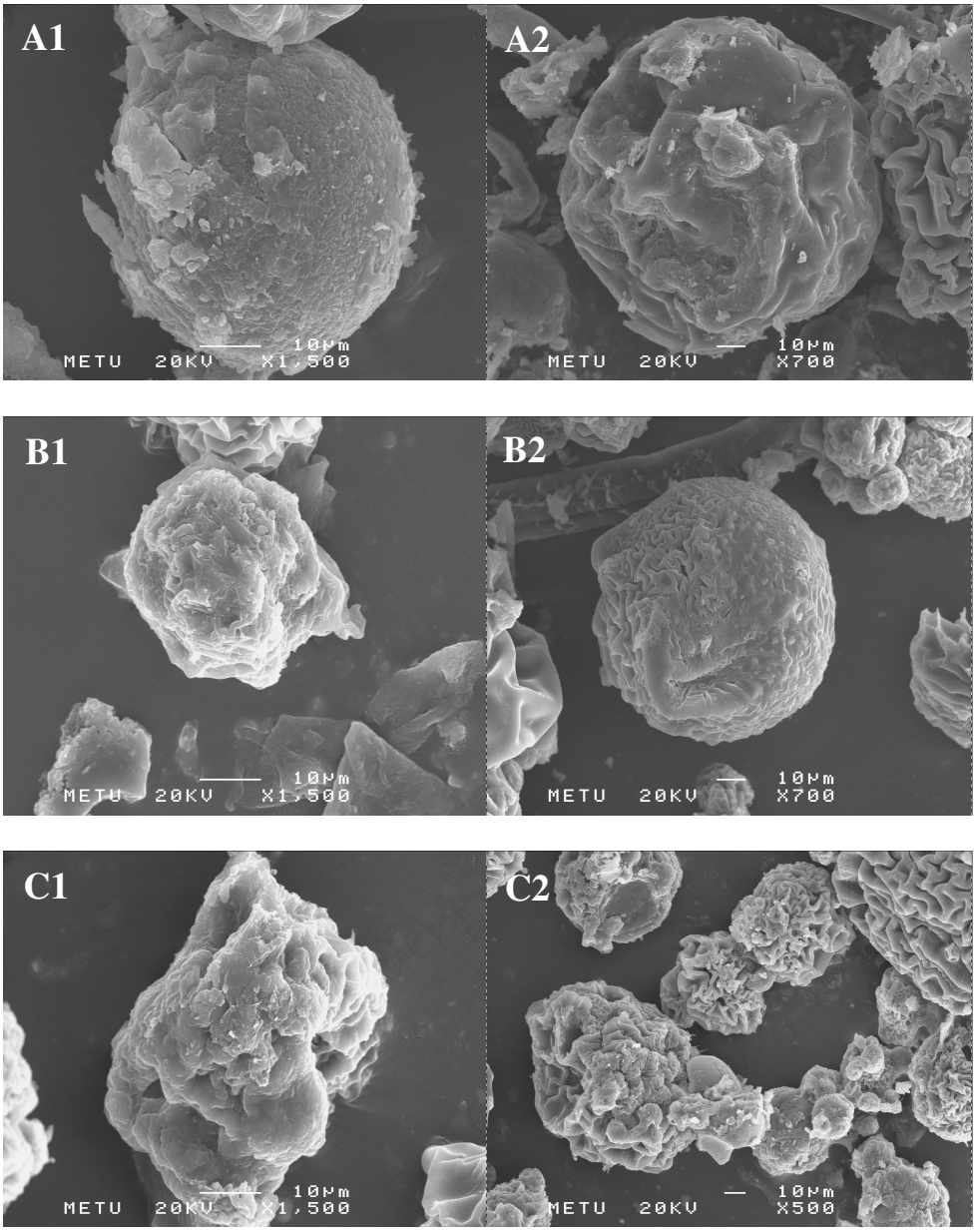

Figure B.2 SEM images of CH:PVP-1:1 microspheres incubated in lysozyme solution for 2 days (A1, x1500; A2, x700), for 15 days (B1, x1500; B2, x700) and for 60 days (C1, x1500; C2, x500)

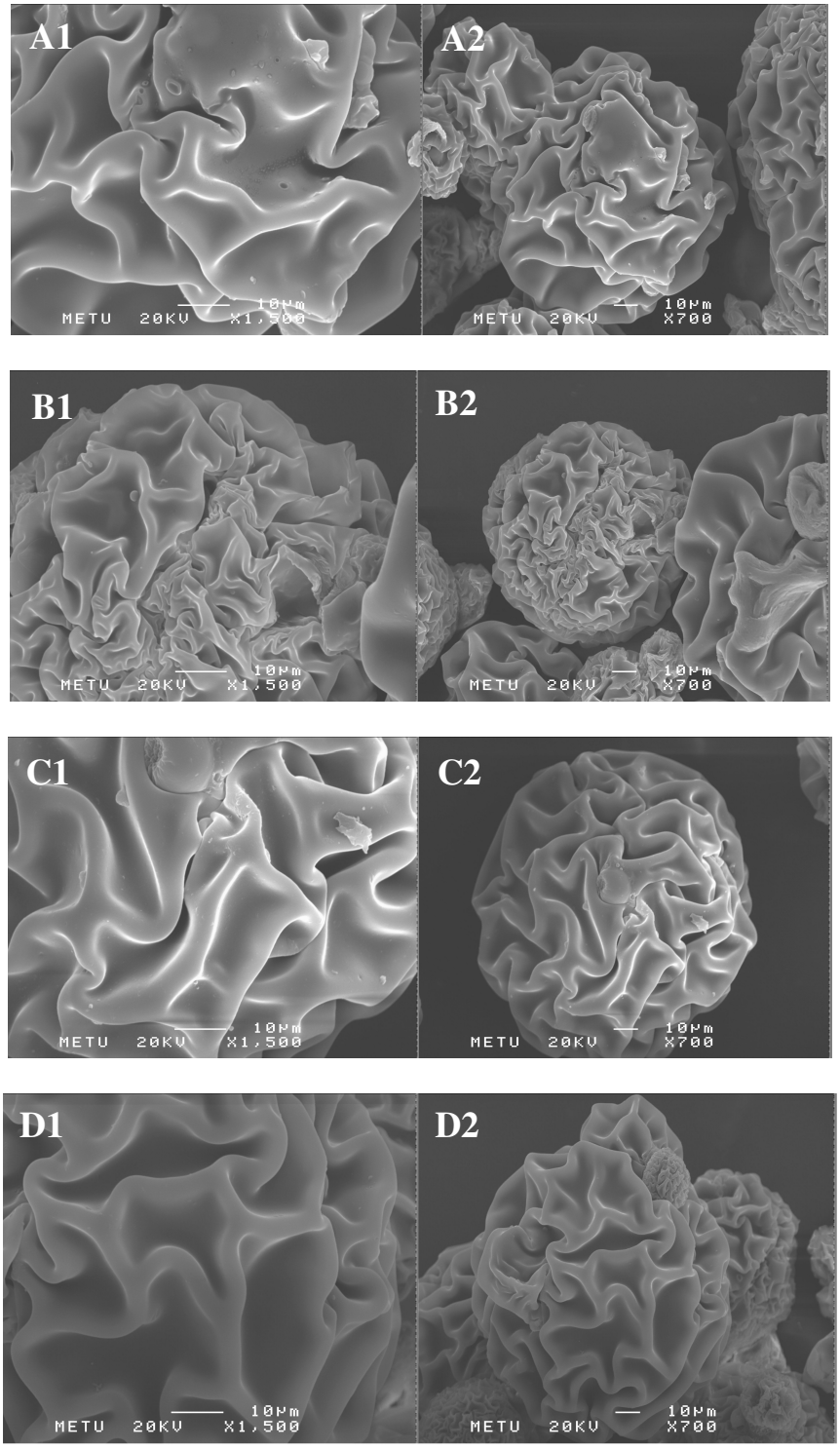

Figure B.3 SEM images of CH (A1, x1500; A2, x700), CH:PVP-1:0,33 (B1, x1500; B2, x700), CH:PVP-1:0,5 (C1, x1500; C2, x700) and CH:PVP-1:1 (D1, x1500; D2, x700)

APPENDIX C

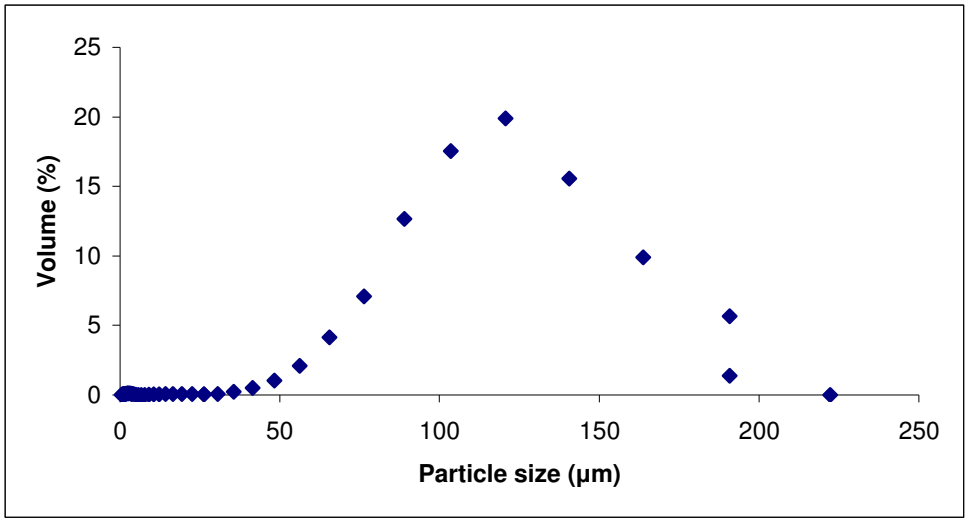

Figure C.1 Size distribution curve of CH:PVP-1:1

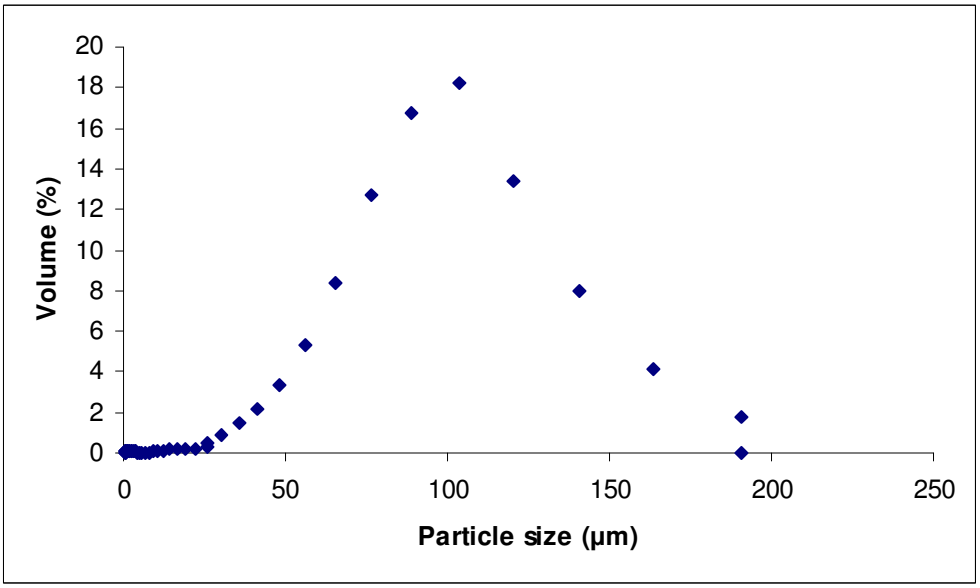

Figure C.2 Size distribution curve of CH:PVP-1:0,5

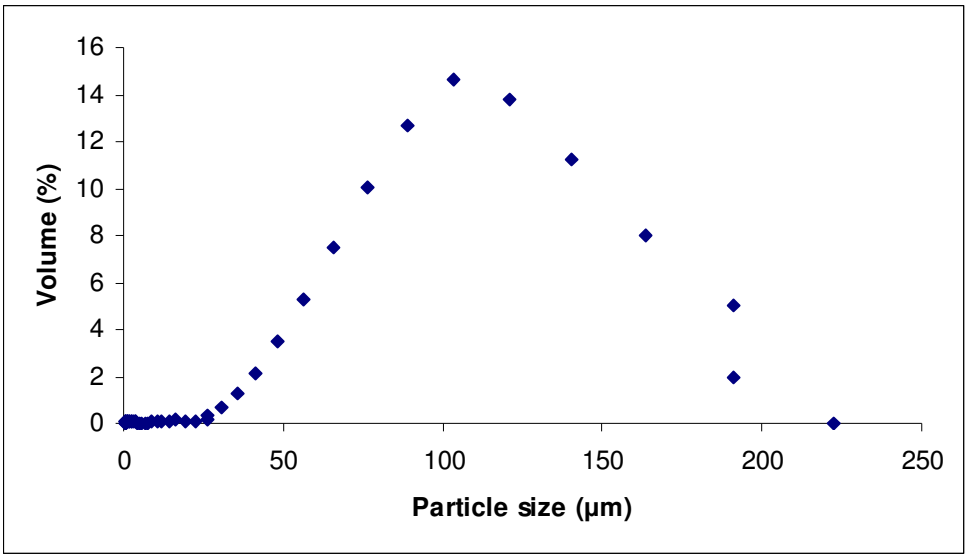

Figure C.3 Size distribution curve of CH:PVP-1:0,33

Table C.1 Size of microspheres

Sample	Diemeter Size (µm)
CH-1.25	136
CH-2.5	97
CH-5.0	90
CH	91
CH:PVP-1:0,33	153
CH:PVP-1:0,5	159
CH:PVP-1:1	170

APPENDIX D

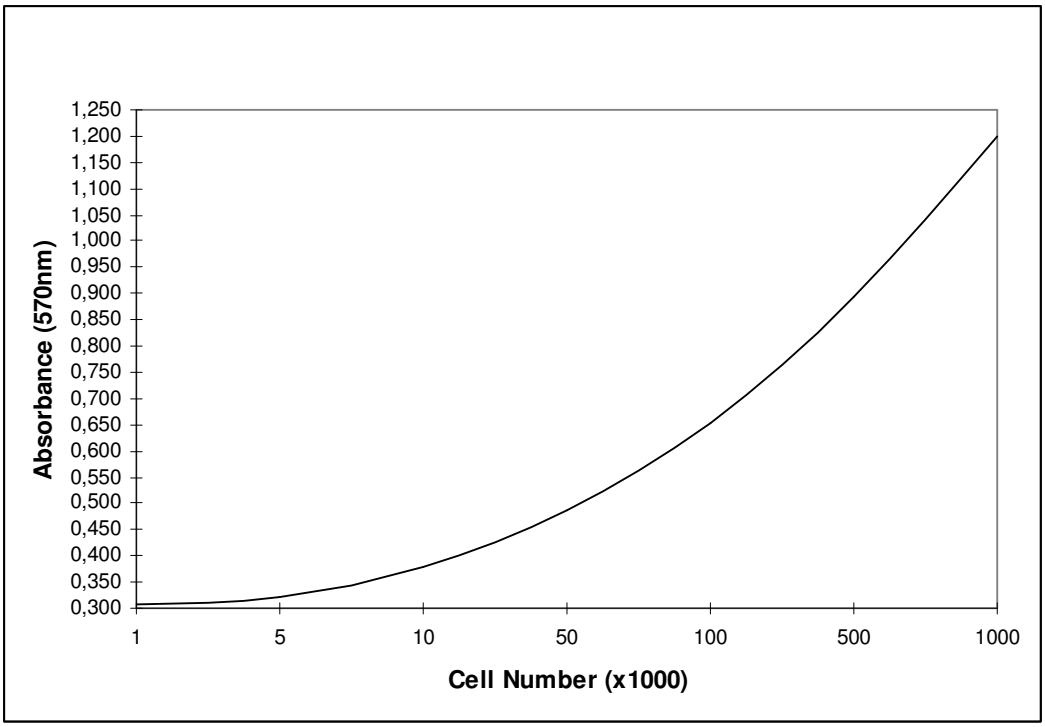

Figure D.1 Calibration curve of MCF-7 MTT test