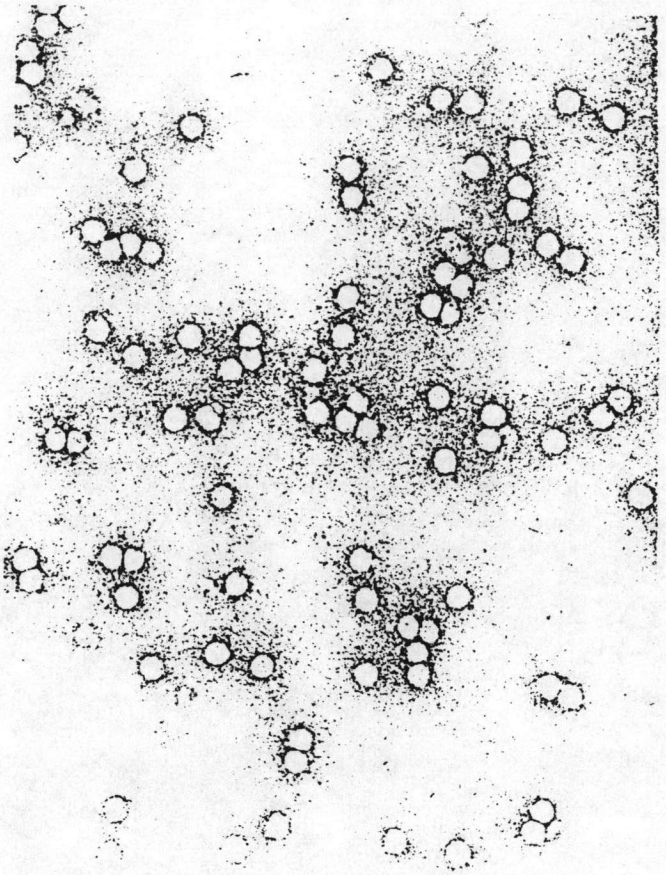
CHAPTER II

LITERATURE REVIEW

Japanese Encephalitis

Japanese Encephalitis (JE) is an arthropod-borne viral infection that occurs mainly in South, East and South-east Asia. It is a leading cause of viral encephalitis in childhood. Although this disease can control in Japan and Korea, but it has still been one of serious diseases with high mortality and grave sequelae in other countries in Asia. JE disease is caused by flavivirus named Japanese Encephalitis Virus (JEV), a member of family Flaviviridae. JEV is a uniformly spherical particle with approximately 45 nm in diameter possessing a lipid envelope which surrounds a densely staining core (Volk, 1991) as shown in figure 1. The genome molecule of JEV is a single-stranded, positive-sense RNA of approximately 11,000 nucleotides in length, 95 nucleotides in 5′ noncoding region, 10,296 nucleotides in a long open reading frame (ORF) and 585 nucleotides in 3′ noncoding region. The ORF is considered to be translated into a polyprotein of 3,432 amino acids. The 5′ region, one-fourth of ORF composes of structural protein genes including envelope protein (E), membrane-like protein (M) and capsid protein (C), which are located near 5′ end in order of C-M-E whereas remaining part of ORF represents nonstructural protein genes such as NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Hashimoto, 1988 and Ma, 1996). The 3′ end does not contain polyadenylated (Poly A) terminus but persume to form stem-and-loop secondary structure. Three structural proteins, the are molecular weight (Mr) predicted to be about 10-14 kDa for capsid protein (C), 8 kDa for unglycosylated membrane protein (M), 18-19 kDa for intracellular glycoprotein precursor to M (pr M) and 53-54 kDa for envelope protein (E). The amphiphilic protein E, comprises spikes of virion, is potently immunogenic. The structure model of E protein is shown in figure 2 (Heinz, 1993).

A)



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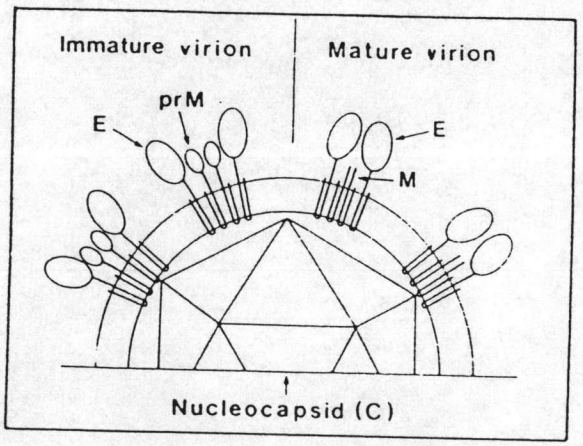


Figure 1 JE virus : A) electron micrograph of JE virus particles , B) schematic model of immature and mature virions

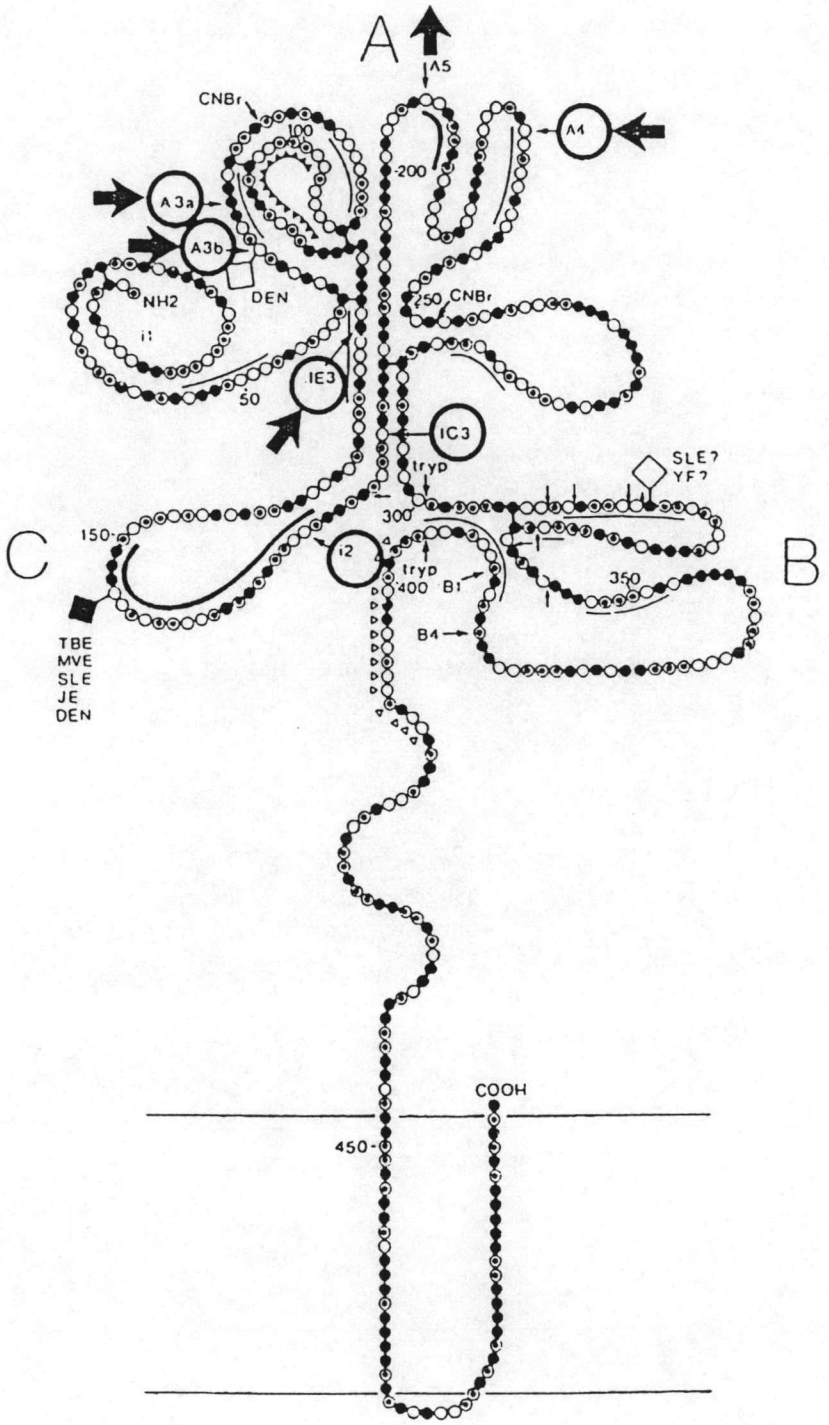


Figure 2 Structure model of E protein

Antigenicity of E protein analysed by monoclonal antibodies expresses multiple epitopes with various cross-reactivity and biological properties. The antibody directed to E protein neutralizes virus infectivity. The M protein spans viral membrane and play an important role in virus assembly. The C protein is associated with core particles and seems to be involved in organizing genomic RNA within virus particles.

The epidemic transmission cycle of JEV in nature is through *Culex tritaeniorhynchus* or related species, the main vector, and swine or avians, the major amplifier vertebrates. However, humans are dead-end hosts due to a low viraemia. Epidemiology of JE in Asia had been described in Table 1.

In Thailand, the first outbreak of acute viral encephalitis found in Chiang Mai in 1969 was associated with JEV. Later, the epidemic area slowly widespreaded and the number of patients increased to exceed 2,000 cases in 1979. (Sangkawibha, 1992). Generally, epidemic of JE was found in rainy season. The mortallity rate was reported to be highest in north followed by north-east areas and relatively low in central and southern Thailand. Children under 14 years were most often influenced by viral encephalitis and approximately 30% of them were serologically confirmed as JE (Igarashi, 1992).

Three JE control strategies have been considered based on transmission cycle of JEV including vector control, swine immunization and immunization of humans. The control of mosquito, JE vector, apparently decreases vector density but effect is just a moment. However, environmental and socioagricultural background support ample vector breeding sites. Although, immunization of swine is carried out in several regions, many problems have been recognized. Therefore, human immunization remains the most reliable control for human JE at present.

Table 1 Reported number of Japanese encephalitis cases (and deaths) in Asia

Year Année	China Chine		Republic of Korea République de Corée		Japan Japon		Viet Nam		Thailand Thaïlande	
1973	89 478	...	769	(157)	70	(27)	1 914	(247)	1 784	(476)
1974	86 900	...	399	(10)	6	(0)	4 246	(925)	1 617	(466)
1975	89 018	...	743	(3)	27	(6)	913	(151)	1 622	(384)
1976	70 319	...	250	(0)	13	(9)	1 789	(542)	1 559	(409)
1977	66 372	...	550	(0)	5	(0)	2 265	(558)	1 738	(422)
1978	51 335	...	560	(0)	88	(21)	1 693	(280)	1 503	(356)
1979	49 287	...	600	(0)	86	(26)	1 688	(387)	2 082	(491)
1980	14 192	(1 793)	107	(4)	40	(15)	1 262	(210)	2 413	(447)
1981	39 985	...	194	...	23	(5)	1 095	(298)	1 562	(257)
1982	31 889	...	1 197	...	21	(4)	2 709	(532)	1 540	(268)
1983	24 260	(2 486)	139	(15)	32	(8)	3 244	(600)	2 084	(371)
1984	26 259	...	...	...	27	(5)	2 431	(407)	1 618	(231)
1985	29 065	...	0	(0)	39	(8)	4 935	(592)	1 980	(267)
1986	18 282	(1 587)	0	(0)	26	(3)	2 889	(515)	1 723	(231)
1987	24 390	(2 283)	3	(0)	37	(7)	3 225	(335)	1 711	(181)
1988	25 123	(2 106)	1	(0)	32	(4)	2 724	(297)	1 587	...
1989	17 098	(1 100)	1	(0)	37	(4)	...	...	1 301	(168)
1990	38 062	(2 626)	1	(0)	54	(8)	916	(95)	1 208	(125)

Table 1 Reported number of Japanese encephalitis cases (and deaths) in Asia (cont.)

Year Année	India Inde		Nepal Népal		Sri Lanka		Myanmar		Bangladesh	
1978	7 463	(2 755)	422	(119)	...	(0)	422	(119)	...	...
1979	2 845	(926)	182	(46)	...	(0)	182	(46)	...	...
1980	3 478	(1 436)	622	(231)	...	(0)	0	(0)	2	...
1981	3 894	(1 167)	54	(16)	...	(0)	0	(0)	0	...
1982	3 515	(1 260)	843	(390)	...	(0)	0	(0)	2	...
1983	1 716	(581)	243	(36)	...	(0)	0	(0)	0	...
1984	3 321	(1 405)	142	(45)	...	(0)	0	(0)	0	...
1985	2 490	(916)	595	(146)	441	(65)	0	(0)	0	...
1986	7 500	(2 617)	1 600	(420)	336	(70)	0	(0)	0	...
1987	3 515	(1 346)	500	(135)	766	(138)	0	(0)	0	...
1988	6 817	(2 407)	1 390	(400)	163	(26)	0	(0)	0	...
1989	6 400	(2 422)	888	(227)	293	(49)	0	(0)	4	...
1990	1 574	(1 291)	365	(102)	387	(43)	0	(0)	1	...

The initial JE vaccine was an inactivated vaccine prepared from infected mouse brains. This vaccine, produced by United States military personnel, was efficacious but it had problem by uncertain rates of adverse reactions in recipients and its mouse brain substrate was a limiting parameter in acceptability and production cost. The adverse reactions such as hypersensitivity reaction consisting of generalized urticaria and angioedema had been disclosed. (Plesner, 1997). A live attenuated JE vaccine strain SA 14-14-2 was developed from virulent SA 14 strain by multiple passages in primany hamster kidney cells (PHK) in China since 1988 (Lee, 1995 and Tsai, 1998). This vaccine is safe, no significant adverse effects and efficacy higher than 95%. It is given in two doses separated by 2.5 months. It currently is in annual spring campaigns to children aging more than 1 year rather than at a specific chronologic age. Current JE vaccine derived from mouse brain is highly purified. It is efficacy, safety and no adverse reaction when used. Inspite of slight antigenic differences among JE virus isolates, Nakayama and Beijing-1, JE vaccine produced by a classical Nakayama strain was effective in preventing over JEV in Thailand (Nimmanitya, 1995). So, Government Pharmaceutical Organization (GPO) in Thailand produced inactivated JE vaccine from Nakayama-NIH in 1986. A schematic diagram of inactivated JE vaccine production is denoted in figure 3 . The vaccine is given in three dosage regimen. The first and second doses are administered at an interval of 1-2 weeks and the third dose is administerd one month after the second dose (Bareztt, 1997). However, research from Handi Biken suggest that Beijing-1 strain had highest potency in producing neutralization antibody in vaccine together with a higher cross-reactivity to heterogenous immunotype strains. Hence, GPO will decide to change vaccine strain from Nakayama-NIH to Beijing-1 strain, in the preliminary stage. Further, the second generation of JE vaccine by using recombinant DNA technology has been developed (Bektimirov, 1990 and Chambers, 1997). Several researches effort to express E-protein gene, but final products give low immunogenicity and unsatisfactory for a vaccine candidate. One

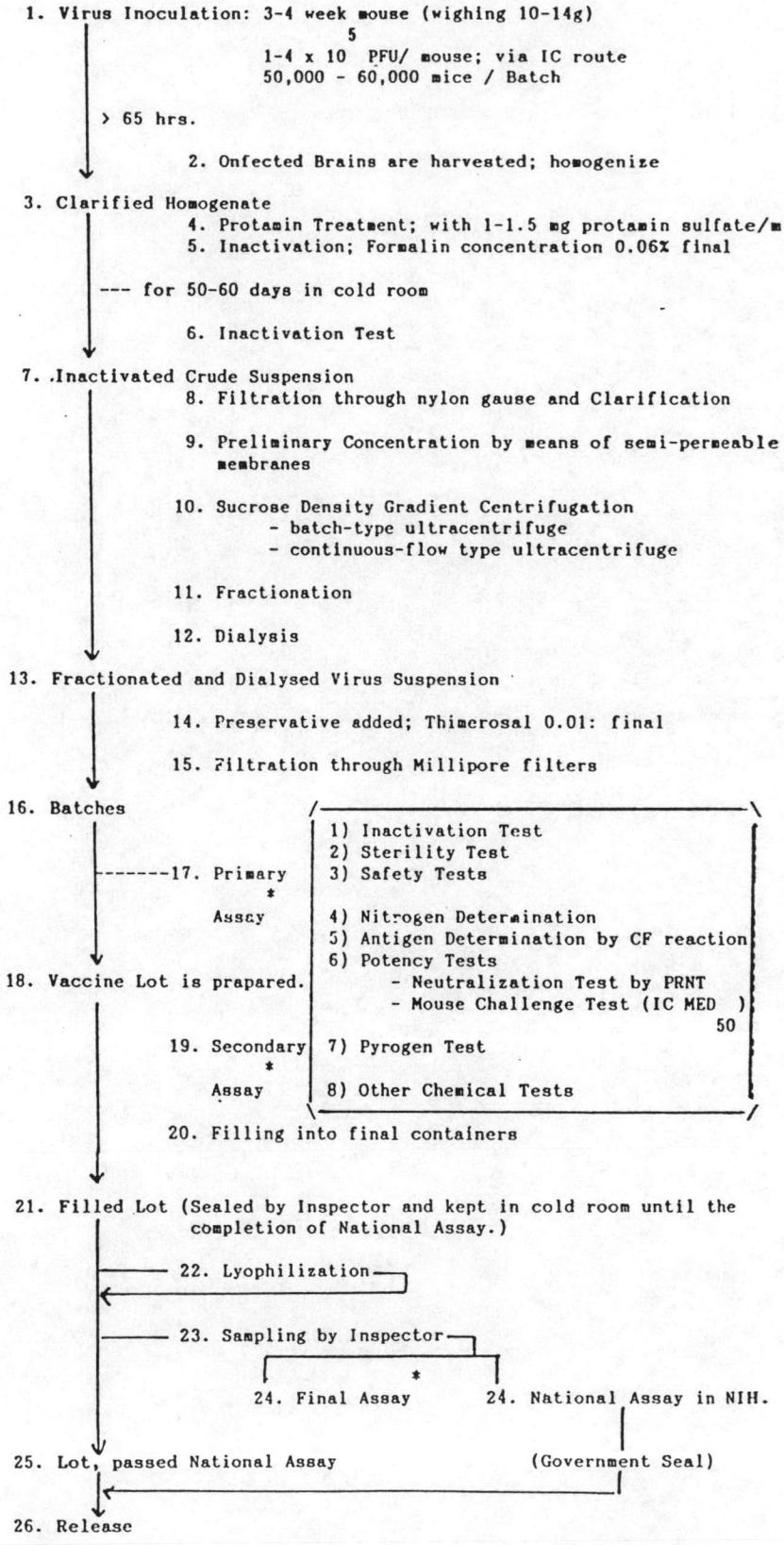


Figure 3 Schematic diagram of inactivated JE vaccine production

approach to reduce this problem is to employ potent adjuvants to enhance effective immune responses.

Vaccine strategies

The immune system can be divided into two functional categories, humoral immunity (HI) and cellular or cell-mediated immunity (CI or CMI). Humoral immunity is influenced by antibody, a bivalent protein molecule that can be cell bound or cell free. Antibodies bind to antigenic determinants which lie on surface of bacteria or viruses and inactivate infectious cells. Antibodies are primarily effective against extracellular infectious components. In the other hand, cell-mediated immunity is mediated by effector cells that destroy infected cells of host by direct cell-to-cell contact or by release of molecules that possess killing activity. So, CI stresses on destruction source of infection and more effective against intracellular infections (Shearer, 1997). However, CI appears to be more complicated. A simplified representation of immune response to a vaccine antigen is illustrated in figure 4.

Both HI and CI require a special type of T cell to initiate and/or augment humoral and cellular responses. A special T cell is known as helper T cell (Th), that produce protein molecules namely cytokines that are responsible for helper effects. The helper T cells are subdivided into two groups, Th1 produced interferon-Υ (IFN-Υ) and Th2 produced interleukin-4 (IL-4), that provided helper activity primarily for HI and CI.

The primary immune response against antigens of infectious agents characteristically involves a cellular effector response followed by a humoral response. The secondary response developes more rapidly and more potent than the primary respone. Several parameters in vaccine design can affect to a dominant humoral or cellular response. Low dose immunization can result in a dominant cellular response

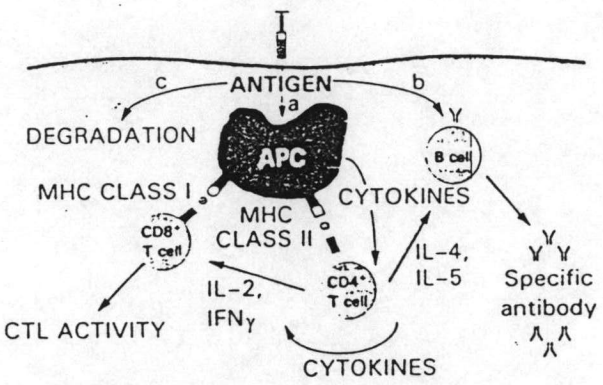


Figure 4 Simplified representation of immune response to a vaccine antigen : a) taken up by antigen presenting cells (APCs), b) bound to surface antibody on B cells , and c) degraded

without appreciable antibody production. However, immunization with higher doses of antigen results in both HI and CI, with HI frequently persisting after CI has declined. Adjuvants can directly affect a dominant HI or CI response, for example alum is known to elicit strong antibody responses, liposomes elicit strong CI and monophosphoryl lipid A (MPL) generates strong and persistent of both HI and CI. Further, type of antigen presenting cells (APCs) can influence dominant humoral or cellular response. APCs of monocytes and macrophages favor dominant CI, while B-cells elicit dominant HI. Ultimately, age of vaccinee may also affect immune potential. The dominant HI may exist in infants and possibly in aged humans. However, several different types of vaccine preparations have been employed for immunization including whole killed organisms, live attenuated viruses, and synthetic peptides. These vaccines can elicit dominant HI, CI or both under appropriate conditions such as route of administration, antigen dose, adjuvant used and cytokine profile of vaccinee.

Vaccine adjuvants

Novel approaches of vaccine delivery system have been developed. Recombinant DNA technology, produced of a variety of subunit vaccines, gives significant advantages over more traditional vaccines. However, a number of these vaccines have been encountered to be nonimmunogenic or weakly immunogenic because of highly purified antigens and small molecular structures (Zhao, 1996). Then, the use of potent adjuvants are required to induce a vigorous immune response.

Adjuvants are described as substances used in combination with a specific antigen that generated more immunity than antigen alone (O′ Hagan, 1997). Some desirable general properties of adjuvants are listed in Table 2.

Table 2 Some desirable general properties of adjuvants

Safety
It must not be carcinogenic, teratogenic, or abortogenic.
The formation of granulomas, local necrosis, hypersensitivity, fever, or autoimmune effects should be avoided.
Nonspecific effects on cell activation, caused by perturbation of cell membranes by surfactants or oils, should be avoided.
It should be biodegradable and preferably have a short half-life.
Specificity
Because most activation signals may be transduced by membrane phospholipase activation, the activity should be targeted to specific cells of the immune system. Such cells may already possess specific receptors, especially if the adjuvant is derived from an infectious agent.
Having a known chemical structure is desirable.
Feasibility and formulation
The preparation should be stable, inexpensive, simple to (reproducibly) manufacture, and have a long storage life.
Presentation of the antigen with adjuvant as particles with multimeric arrays of antigen is advantageous.

Mechanism of action of adjuvants

Adjuvants can also perform in one or more five mechanisms as concluded in Table 3. (Gupta, 1992 and Cox, 1997).

1. Immunomodulation

This mechanism refers to ability to modify cytokine network. Immunomodulation may result in a general upregulation of entire immune system, that most commonly results in upregulation of certain cytokines and concomittantly down regulation of others. Two major subsets of T cell, Th 1 and Th 2 have involved in this mechanism. Th 1 responses typically induce complement fixing antibody and strong delayed-type hypersensitivity (DTH) reactions. Th 2 responses result in high circulation and secretory antibody levels. However, Th 1 and Th2 responses are mutually impediment.

Immunomodulatory adjuvants not only lead to increase immune response but also determine IgG isotype which other immunoglobulins and how much T cells directed, so cell mediated immunity is generated.

2. Presentation

Presentation means ability to preserve conformational integrity of an antigen and to present to suitable immune effector cells. This ability happen when an adjuvant is able to interact with an antigen in such a way that conformational epitopes are more effectively maintained. Hence, the major advantages of antigen presentation are an improved amount of conformationally relevant antibody such as neutralizing antibody , an increared affinity of antibody and duration of immune response.

Table 3 Mode of adjuvant action

Action	Adjuvant type	Benefit
1. Immunomodulation	Generally small molecules or proteins which modify the cytokine network	Upregulation of immune response. Selection of Th1 or Th2
2. Presentation	Generally amphipathic molecules or complexes which interact with immunogen in its native conformation	Increased neutralizing antibody response. Greater duration of response
3. CTL Induction	• Particles which can bind or enclose immunogen and which can fuse with or disrupt cell membranes	Cytosolic processing of protein yielding correct class 1 restricted peptides
	• w/o emulsions for direct attachment of peptide to cell surface MHC-1	Simple process if promiscuous peptide(s) known
4. Targeting	• Particulate adjuvants which bind immunogen. Adjuvants which saturate Kupffer cells	Efficient use of adjuvant and immunogen
	• Carbohydrate adjuvants which target lectin receptors on macrophages and DCs	As above. May also determine type of response if targeting selective
5. Depot generation	• w/o emulsion for short term	Efficiency
	• Microspheres or nanospheres for long term	Potential for single-dose vaccine

3. Induction of cytotoxic T-lymphocyte (CTL) responses

The CTL induction responses usually require that antigen be processed within cytosol cell (endogenous pathway) where peptides become incorporate within close-end groove of major histocompatibility class 1 (MHC-1) molecule and are then expressed on cell surface.

An adjuvant to be used for CTL induction must facilitate incorporation or persistence of appropriate peptide into MHC-1. The adjuvant interact with cell membrane so that antigen associated with adjuvant is deposited within cytosol cell. Although most cells express MHC-1, the most effective target cell for CTL induction is an antigen presenting cells (APCs) and most probably a typically dendritic cells (DC).

4. Targeting

It defines as ability to deliver an antigen to immune effector cells, generally via APCs. This form of adjuvant activity may not modify immune response type but rather influence an efficiency of generation of immune response. There are several means in which an adjuvant can achieve this effect. The most common is to interact antigen to form multimolecular aggregates, which encourage uptake by macrophages and DC, and deliver antigen to APCs. Adjuvants with this property are called particulate adjuvants.

5. Depot generation

This mechanism can be divided into two groups, a short term and a long term depot. Short term depots generate when antigen is entrapped at site of administration and thus cannot be destroyed by liver clearance. This term is typified by aluminium salts and water-in-oil (w/o) emulsions.

The most common approach to achieve a long term depot is using synthetic polymer to produce microparticles. The microparticles with more than 10 μm in size are maintained at site of administration and encapsulated antigen will be released when polymer biodegradation. Long term depots give either continuous or pulse release. It concludes that short term depots similarly increase efficiency whereas long term depots offer opportunity for single-dose vaccines.

Classification of adjuvants.

The successful development of adjuvants requires consideration of a number of issues. The characteristics of an ideal vaccine adjuvant are shown in Table 4 . The safety is the most substantial issue to be considered. Other considerable issues are including stability, ease to produce, a wide range applicability and cost. Moreover, an ideal adjuvant of vaccine should be capable of being administered with a wide range of antigens by a variety of different routes, such as oral, parenteral or intranasal.

There are a great number of different criteria which can be used to classify vaccine adjuvants. However, based on particular system, adjuvants are classified into two groups, nonparticulate and particulate adjuvants, as briefly described below.

1. Nonparticulate systems

Nonparticulate adjuvants are adjuvants where activity does not depend on any particulate or multimeric nature. In some instances, they can afford good immune responses, generally, through immunomodulation action but some improve targeting. The characteristics of principal and other nonparticulate adjuvants are summarized in Table 5. The most interest in nonparticulate adjuvants are lipopolysaccharide (LPS), muramyl dipeptide (MDP) and derivatives, and non-ionic block copolymers.

Table 4 The characteristics of an ideal vaccine adjuvant

Biodegradable and biocompatible
Should not be toxic, carcinogenic, teratogenic or abortogenic
Non-antigenic and not immunologically cross-reactive with tissue antigens
Induce a minimum of injection site reactogenicity
Simple well defined chemical structure
Induce a minimum of non-specific effects on immune system
Acceptable for administration to man
Safe to administer to young and immunocompromised individuals
Effective for peptide, protein, polysaccharide and DNA
Effective after a single dose
Induce both humoral and cell-mediated immunity
Capable of being administered orally
Induce systemic and mucosal immunity
Promote antigen uptake by lymphoid tissues
Stable formulation which is inexpensive to manufacture
Can be manufactured reproducibly on a large scale
Good shelf-life, preferably without refrigeration
Easy to mix with antigen or combination of antigens

Table 5 Characteristics of nonparticulate adjuvants

Adjuvant	Immunomodulation	Targeting	Presentation	CTL	Comments
MDP-hydrophilic	Strong Th2	–	–	–	Use in w/o emulsions
MDP-Lipophilic	Strong Th1	–	–	–	Use in o/w emulsions
Non-ionic block copolymers	?	– or +++[a]	+++	–	Use in w/o or o/w emulsions
Saponins	Strong Th1 . Th2	–	–	+	Form ISCOMs. Use with liposomes, MPL
Lipid A (MPL)	Strong Th1	–	–	–	Use with o/w emulsions, liposomes, saponins
Cytokines	Various	–	–	–	Use preferably with some particulate adjuvant
Carbohydrate polymers	Mod Th1. IL-1 induction	+++	–	–	Preferably conjugate?
Derivatized polysaccharides	?	+++	–	–	

Table 5 Characteristics of nonparticulate adjuvants (cont.)

Adjuvant	Action	Comments
AvridineDDA	Th1 induction Presentation (in liposome or o/w emulsion)	Unacceptable toxicity
CWS(cell wall skeleton)	Th1 induction?	Use with MPL in o/w emulsions
DHEA,(dehydroepi- androsterone)	Th1 induction	Administration difficult
Vitamin D3	Th2, secretory IgA induction?	Administration difficult
TDM(Trehalose dimycolate)	Th1 induction	Administration difficult. Toxicity unacceptable
P_3CSS	Targeting. Potent CTL induction	Potentially toxic
Poly I:CPoly ICLC	Both Th1 (γIFN) and Th2 (IL-4) induction	Poly I:C toxic
Poly A:U	Th2 induction (IL-6)	

Table 6 Characteristics of particulate adjuvants

Adjuvant	Immunomodulation	Targeting	Presentation	CTL Induction	Depot
Aluminium salts	Strong Th2. IgE	+	–	–	+ST[a]
W/o emulsions	Weak Th1 and Th2	–	–	– or +++[b]	+-+ST
O/w emulsions	Weak Th1 and Th2	+	+++	–	–
ISCOMs[c]	Strong Th1 and Th2	+++	++++	++++	–
Liposomes	–	++	+++	++	–
Microparticles					
<10 μm	–	++++	–	-	-
>10 μm	–	–	–	–	+++LT[c]
Calcium salts	–	+	–	–	+ST
Proteasomes/virosomes	–	++	+++	–	–
Stearyl tyrosine	Mod Th1 and Th2	–	–	–	+ST
γ-Inulin	Mod Th1	–	–	–	–
Algammulin	Mod Th1 and Th2	+	–	–	+ST

[a]ST short term (≤ 2 week); [b]Good CTL response for externally applied peptide only; [c]LT, long term (weeks to months)

1.1 Lipopolysaccharide

LPS is an amphipathic molecule with three covalently linked regions, o - specific polysaccharide, core oligosaccharide and lipid A (Raetz, 1990). Lipid A , a disaccharide of glucosamine with two phosphate group and five or six fatty acid chains, contains most of biological activities not only adjuvanicity but also pyrogenicity and toxicity (Luderitz, 1982). Removal of $1'$ phosphate group gives a relatively nontoxic $4'$ – monophosphoryl lipid A (MPL) which retained most of biological activities of lipid A including adjuvanicity but less pyrogenic and relatively nontoxic (Rudbach, 1990 and Ulrich, 1995). Lipid A is an adjuvant for both humoral and cell-mediated immunity. It can augment immune response to both protein and polysaccharide antigens. The mode of action has been mainly considered as a T-cell independent polyclonal B-cell mitogen. The adjuvanicity for polysaccharide antigens is due to regulation of T cells particularly T-suppressor cells. MPL, detoxified preparation of lipid A, can act as lipid A and is good candidate for immunological adjuvant for human vaccines.

1.2 Muramyl dipeptide and derivatives

N- acetyl muramyl-L-alanyl-D-isoglutamine or muramyl dipeptide (MDP) is an adjuvant active component of a peptidoglycan extracted from Mycobacterium. The mechanism of adjuvant action of MDP is complicated. It can activate several cell type of immune system including macrophages, polymorphonuclear leucocytes, mast cells, platelets, endothelial cells and fibroblasts. Further, it induce carrier-specific helper T-cell function. MDP can augment both humoral and cell-mediated immunity depending on form in which it is given. However, MDP itself is considered unacceptable as an adjuvant for human use due to pyrogenicity (Riveau, 1980). The derivatives of MDP have been modified to maintain adjuvanicity with minimum toxicity and nonpyrogenicity such as murametide, threonyl – MDP and murabutide

(Byars, 1987). Threonyl – MDP has an adjuvant effect, nonpyrogenic and does not stimulate macrophage – mediated carbon clearance. So, it has been developed as Syntex Adjuvant Formulation-1 (SAF-1), a threonyl –MDP in a squalene-pluronic polymer emulsion, by Allison and Byars (1990). SAF-1 induced both humoral and cell-mediated immune response. It has been described as being efficacious without any side effects. Thus, SAF-1 seems to be one of adjuvant that safety and adjuvanicity towards a broad range of antigens.

1.3 Non-ionic block copolymers

These polymers most typically comprise a region of hydrophobic polyoxy propylene (POP) sided by regions of polyoxyethylene (POE). The length of POP and POE chains affects adjuvant activity (Hunter, 1991). The nonionic block copolymers influence isotype as well as intensity of immune response, producing an increased proportion of highly protective IgG_2 isotype. However, other mechanism including presentation of amphipathic molecule and targeting to APCs can be observed. Combination with LPS produced synergistic adjuvant effects with acceptable toxicities (Takayama, 1991).

2. Particulate systems

The substances which exist as microscopic particles have an adjuvant activity. The particulate adjuvants as denoted in Table 6 are advantages when antigen is able to be adsorbed or incorporated or encapsulated in particles. The detail of particulate adjuvants that most commonly used are described below.

2.1 Aluminum compounds

Aluminum compounds or alum such as aluminum hydroxide or phosphate

are approved for use in vaccines by USA Federal Drug Administration. They have been licenced for human use since 1920's. They mainly act as adjuvant activity by depot for depot formation, allowing slow release of antigen and prolong interaction time between antigen and antigen presenting cells and lymphocytes. However, other mechanisms such as induction of Th2 responses and targeting are involved adjuvant effect. Alums are not always effective, they augment only humoral immunity but not induce in cell-mediated immunity (Nicklas, 1992). The aluminum compounds are considered relatively safe, inexpensive and simple to formulate. However, local reactions following administration including erythema, subcutaneous nodules, contact hypersensitivity and granulomatous inflammation are observed (Mark, 1995).

2.2 Emulsions

Freund's complete adjuvant (FCA), a water – in - oil emulsion contain immunostimulatory compounds from Mycobacterium tuberculosis, is one of the most potent adjuvants. It is used extensively to enhance immune response in animals but it is too toxic to be used in humans. The side effects include abscess formation and inflammation at administration site, fever, severe pain, permanent organ injury, granulomatous proliferation, induction of autoimmune diseases, amyloidosis, adjuvant arthritis and hypersensitization (Steiner, 1960). The water-in-oil emulsion contains only mineral oil and Aracel A (without mycobacteria) is known as Freund's incomplete adjuvant (FIA). The mechanism of action of FIA is to depot formation at site of administration and slow release of antigen with stimulation of antibody-producing cells. However, FIA does not significantly enhance cell-mediated immunity when absence of mycobacteria (Herbert, 1968).

FIA uses as adjuvant in many veterinary vaccines, but it is not currently used in humans because of its side effects including local reactions such as granuloma, sterile

abscesses and cysts formation and inflammation. The other side effects include systemic reactions and possibly carcinogenicity are observed (Gupta, 1993).

2.3 Immunostimulating complexes (ISCOMs)

ISCOMs are seen as characteristic cage-like structures of 30-40 nm in size. They are relatively stable, but noncovalently bound, complex of saponin adjuvant Quil-A, cholesterol, and amphipathic antigen in a molar ratio of approximately 1:1:1 (Bengtsson, 1996). The amphipathicity of antigen is a mainly factor for interaction with Quil-A/cholesterol matrix. This approach can stimulate both humoral and cell-mediated immune response towards amphipathic antigens such as measles, rabies and influenza. Moreover, they have been shown to induce cytotoxic T- lymphocytes (CTL induction) (Takahashi, 1990). Furthermore, oral administration of ISCOMs MHC-I restricted CTLs is found in mesenterial lymph nodes and spleen and a specific Ig A response can be augmented. However, their toxicity are not clear, so they are only used in veterinary vaccines in the present. In addition, this technology will further study and may be accepted for human use in future.

2.4 Liposomes

Liposomes are particles made up of concentric lipid membranes containing phospholipids and other lipids in a bilayer configuration separated by aqueous compartment. The size varies from 30 nm to several micron. They act as a potent and nontoxic immunological adjuvant with both protein and carbohydrate antigens. The adjuvanicity relies on the number of lipid bilayers, charge, composition and preparation technique.

Liposomes enhance both humoral and cell-mediated immune response (Cohen, 1994 and Phillips, 1996). They act as a vehicle for antigens not only clearance of

antigens incorporated into liposomes markedly prolonged but liposomes may also ensure that a certain amount of antigen is made available for a single antigen presenting cell at a time following phagocytosis. So, liposomes enhance antigen presentation to macrophage. Further, CMI is stimulated due to hydrophobic nature of liposomes. Moreover, mucosal response can be augmented by liposomes, so they have also been used as oral adjuvants (Gregoriadis, 1995).

Liposomes, themselves, are poor immunogens because they consist of self-component, nonimmunogenic and biodegradable lipids. In addition, they are biodegradability, biocompatibility, no tissue reaction, nontoxicity, stability and reproducibility in production. Therefore, the behavior and properties of liposomes can effort towards application of system as immunological adjuvant or vaccine carrier (Cryz, 1996). The first liposome-based vaccine has been licenced for use in Switzerland as Epaxal Berna®, a vaccine against hepatitis A.

2.5 Biodegradable microparticles

The adjuvant effect achieved through the association of antigens with polymeric microparticles have been developed. Microparticles, small solid particles ranging from 1 to 1,000 μm, are made from biocompatible and biodegradable polymer including polylactide-co-glycolide, polyphosphazene, polyanhydride and natural substances such as gelatin and albumin. They can entry into lymph nodes and provides a high local concentration of antigen over an extended time period. Further, they also promote interaction of encapsulated antigens with APCs such as macrophages. In addition, they also exert an adjuvant effect for cell-mediated immunity, including induction of CTL responses after both systemetic and mucosal administration (Powell, 1996). The other mechanism involved is long term depot. They can control release rate of entrapped antigens and, ultimately, enable offer the development of single

dose multi-release vaccines (Morris, 1994). Microparticles have been suitable used for both oral and parenteral administration. The advantages of biodegradable microparticles for vaccine adjuvants are denoted in Table 7.

However, properties of microparticulate system, particularly particle size influence adjuvant effect. Small particle with size less than 10 μm allows uptake by macrophages and transport to lymphatic whereas large particle with size greater than 10 μm can be sequestered but not translocated and act as long term depot. Therefore, smaller particles show significantly more immunogenic than larger particles (Eldridge, 1991).

Table 7 The benefits of biodegradable microparticles for vaccine adjuvants

Safety : biocompatible and biodegradable polymers
Controlled release may enable development of single dose vaccines
Many antigens can be entrapped simultaneously in microparticle
Microparticles may be administered by mucosal routes including oral delivery
Antigens are protected from degradation in intestine
Antigens are targeted to lymphoid tissue
Microparticles induce serum and secretory antibodies
Microparticles induce cell-mediated immunity
Freeze-dried formulations with enhanced stability for entrapped antigen
Large scale manufacture of microparticles has already been achieved
Acceptable for administration to human (poly (lactide-co-glycolide)

Microparticles

Microparticles are small solid particles which size ranging from 1 to 1,000 μm. They are commonly described as a container or vessel for enclosing and protecting

certain amount of drugs or other substances. They consist of macromolecular materials and can be used therapeutically as drug carriers or adjuvants in vaccines, in which active substance is dissolved, entrapped or encapsulated and to which active principle is attached or adsorbed. The microparticles can be classified into two main groups. One is reservoir type with active material formed in core and coated by a polymeric membrane. This type is generally known as microcapsules. The other is monolithic type with active substance evenly dispersed throughout polymeric matrix. This type is called microspheres (Aguado, 1993). The core material is a liquid or solid containing one or more drugs enclosed in coating. The core may also be referred to as nucleus or fill and coating as wall or shell. Various structure of microparticles can be obtained as illustrated in figure 5.

Function of microparticles

The microparticles have a capability of modifying and improving the apparent shape and properties of a substance. More specifically, the microparticles have an ability to preserve a substance in the finely divided state and release the substance as occasion demands. There are many reasons why drugs and related substances have been microencapsulated (Kondo, 1979 and Deasy, 1984) such as

1. Conversion of oils and other liquids to solids

When a liquid substance is microencapsulated, a solid fine powder is obtained. However, the internal phase is still liquid and maintain the superior reactivity of a liquid phase. Therefore, microencapsulation improves ease of handling of liquid reactants and permits chemical reactions to take place at a specified time when the capsule is ruptured.

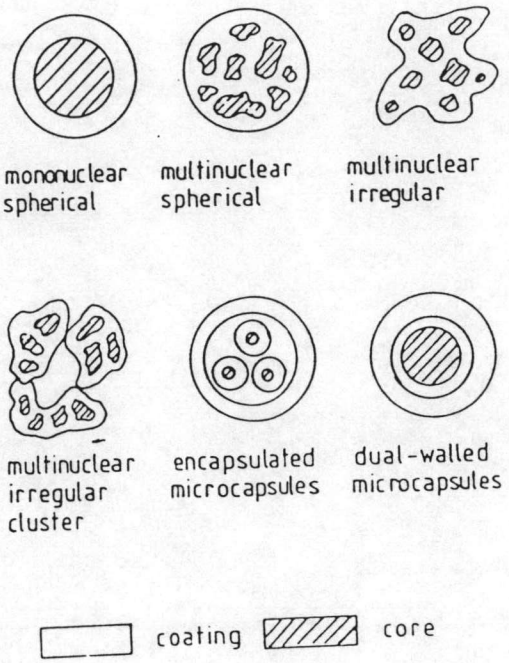


Figure 5 Typical structures of microparticles

2. Change of weight or volume

The weight of a substance can be increased by microencapsulation and the volume can also be increased by forming it into an air-containing capsule or hollow capsule. Thus, a dense solid may be converted through encapsulation to a product which will float on water.

3. Delay of volatilization

An encapsulated volatile substance can be stored for a long time without substantial evaporation because the contained volatile substance is hardly released at room temperature.

4. Separation of incompatible materials

The microparticles have an ability to prevent chemical reaction between two reactive species through physically separating the components. When one of two incompatible component is microencapsulated and mixed with the other component, they can be stored in a long time.

5. Protection of core materials against atmospheric effects

The microparticles have an ability to protect a substance from environmental actions of moisture, oxygen, light and/or heat.

6. Improvement of flow of powders

National Cash Register reported that the flow properties of vitamins such as thiamine hydrochloride, riboflavin and niacin with iron phosphate could be improved by microencapsulation prior to compression into tablets. The choice of suitable coating material should improve the compaction and subsequent disintegration of encapsulated drugs from tablets.

7. Controlled release

A core substance contained in a microparticle may be released instantaneously or gradually over a period of time. For instantaneous release, the mechanical means such as compression, crushing, deformation, friction, burning and melting under heat or chemical means such as attack by enzyme and dissolution and extraction with solvent or water may occur. It is also possible to obtain instantaneous release by incorporation of a swelling agent into the core substance or by an electromagnetic method using discharge or magnetic force (Hassan, 1992). In pharmaceutics, the gradual release of core substance is obtained. When a water-soluble core drug is microencapsulated with a water-insoluble wall material, the release of active drug to an external aqueous phase is controlled by rate of diffusion and dissolution. The type of wall material, thickness, hardness and layer-structure of wall, drug loading, polymer molecular weight and particle size may affect rate of release. Furthermore, most microparticles have a very small size, they have a very large surfure area per unit weight. In most medicinal microparticles, active materials are released through the semipermeable characteristic of the wall. Hence, controlled release is frequently accomplished through a diffusion controlled process.

Further, microencapsulation has been employed to disguise the odor and unpleasant taste of drugs. However some drugs such as ferrous sulfate and potassium chloride have been microencapsulated to reduce gastrointestinal tract irritation.

Limits of microparticles

Although microparticles have much advantages, there are still many difficulties to be resolved. The problems frequently encountered include incomplete or uneven coating deposition, clumping of particles, unsatisfactory or nonreproducible drug release

and scale-up difficulties. Each microencapsulated product requires an individual design approach, and there is no one methodology that is suitable in all cases. However, satisfactory toxicological data on polymers and other materials for use in microencapsulated medicines for use in humans must be available before being authorized for clinical trials and marketing.

Microencapsulation process

A number of microencapsulation methods have been developed. The technique to prepare microparticles is dependent upon nature of drug and polymer, intended use and duration of therapy. The microencapsulation method used which has the requirement are following described :

1. The stability and biological activity of drug should not be adversely affected during encapsulation process or in final microparticle product.
2. The yield of microparticles having required size range, ideally < 125 μm and drug encapsulation efficiency should be high.
3. The microparticle quality and drug release profile should be reproducible within specified limits.
4. The micropaticles should be produced as a free-flowing powder and should not exhibit aggregation or adherence.

However, different methods of microparticle preparation are described below.

1. Coacervation – phase separation procedures (Bakan, 1990)

Encapsulation by coacervation is an uncomplicated process. Coacervation is the separation of a macromolecular solution into two immiscible liquid phases, a dense coacervate phase, which is relatively concentrated in macromolecules, and a dilute

equilibrium phase. The typical steps involved in a coacervation process is shown in figure 6. There are two types of coacervation. Simple coacervation involves the use of only one macromolecule such as gelatin and involves removal of the associated water from around the dispersed macromolecule by agents with a greater affinity for water such as alcohols and salts. The dehydrated molecules of polymer tend to aggregate with surrounding molecules to form the coacervate. Simple coacervation is induced by a change in conditions which results in molecular dehydration of the macromolecules. This may be achieved by the addition of a nonsolvent, the addition of micro-ions, or a temperature change, all of which promote polymer-polymer interactions over polymer-solvent interactions. The typical diagram for encapsulation using simple coacervation is shown in figure 7. Complex coacervation involves the use of two or more macromolecules which opposite charge such as gelatin and acacia or carbopol and gelatin (Elegakey, 1983). The coacervation is accomplished mainly by electrostatic interactive forces between two or more macromolecules rather than by dehydration. A typical diagram for encapsulation using complex coacervation is shown in figure 8. However, there are a number of limitations of coacervated micropaticles. The microparticles can be produced only at specific pH values. The pH limitation can be overcome to some extent by addition of water-soluble nonionic polymers, such as polyethylene oxide or polyethylene glycol. The presence of a small amount of these polymers allows microencapsulation to occur over an expanded pH range. Further, coacervation process requires stabilization by cross-linking agents or heat. The retention of the encapsulant depends on the extent of cross-linking. Glutaraldehyde and formaldehyde are commonly used as cross-linking agents. However, the use of these toxic chemicals in pharmaceutical products is careful. Both chemical cross-linking agents and heat may be harmful to the encapsulant materials such as chemically-labile and thermo-labile substances and live cells. In addition, coacervation method tends to produce particles that are agglomerated, there is difficulty in mass production, the

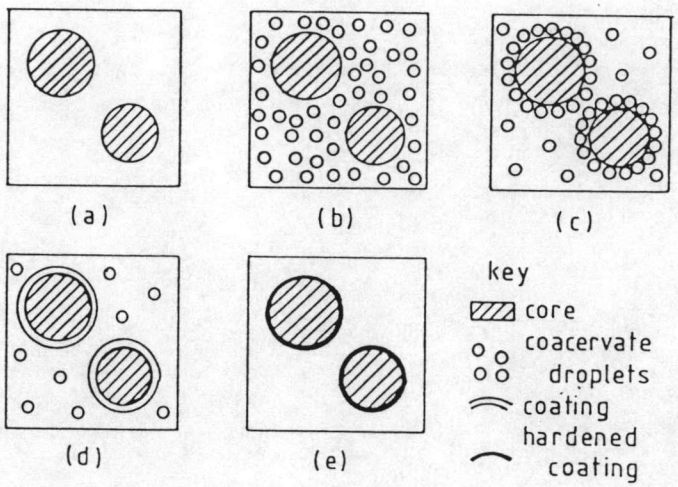


Figure 6 Typical steps in a coacervation method of microencapsulation : a) core particles dispersed in solution of polymer by agitation , b) coacervatin visible as droplets of colloid - rich phase induced by one or more agents , c) deposition of coacervate droplets on surface of core particles , d) mergence of coacervate droplets to form the coating , e) shrinkage and crosslinking of the coating to rigidize it as necessary

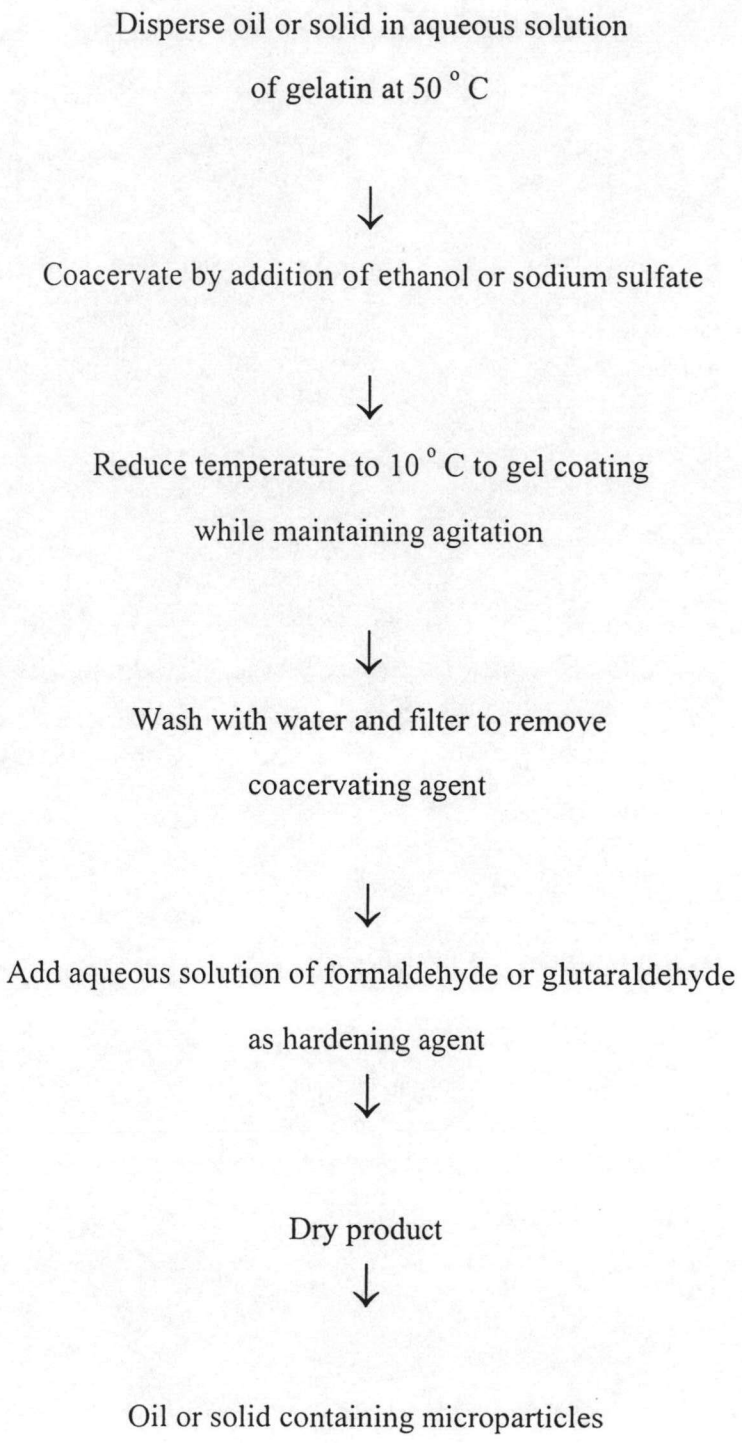


Figure 7 Schematic diagram of a simple coacervation process

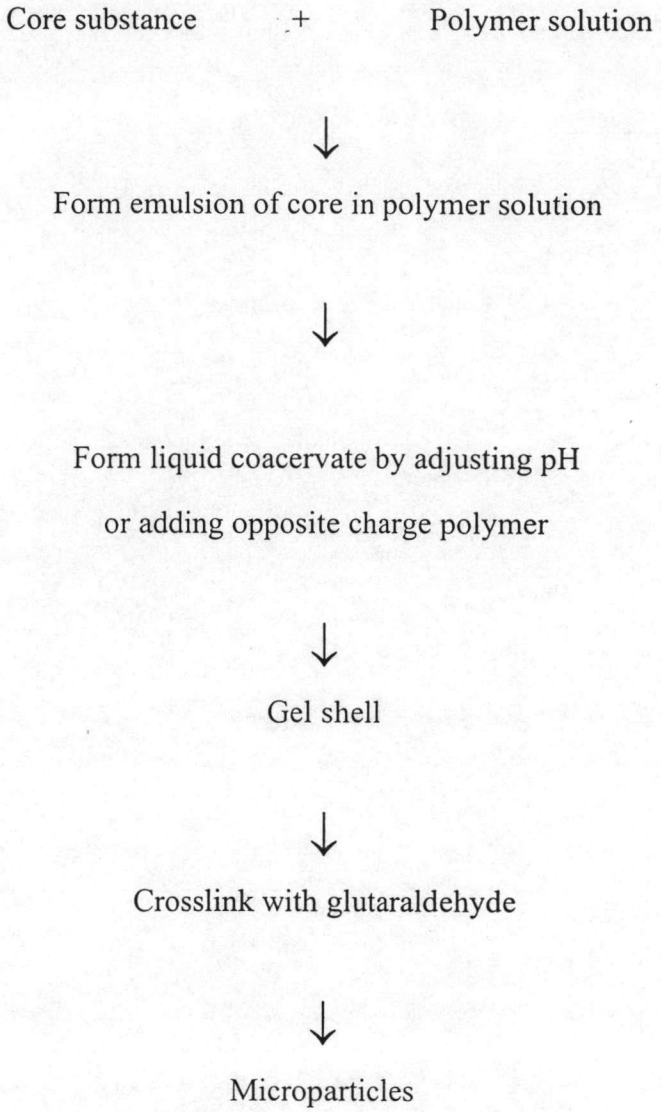


Figure 8 Schematic diagram of encapsulation process based on complex coacervation

method requires large quantity of organic solvent , and it is difficult to remove residual solvent from final microparticle products.

2. Spray drying technique

Spray drying has been used as the important means of microencapsulation in a wide range of materials. The core drug and coating polymer are dissolve in a suitable solvent, aqueous or nonaqueous, or drug may suspend in polymer solution like suspension or drug may be dissolved or suspended within an emulsion or coacervate system, which is then atomized and the solvent dried off using heated air in a spray dryer. A flow diagram of spray drying microparticle manufacturing method is shown in figure 9. The size of microparticle is controlled by rate of spraying, feed rate of polymer - drug solution, nozzle size, size and temperature of drying and collecting chambers. The particle size is commonly described as being less than 10 μm and size distribution is usually monodispersed with a Gaussian distribution because the geometries of atomizers and viscosity of nebulized solutions are almost uniform (Bafler, 1994). Furthermore, residual level of organic solvent are less than the level from emulsification-solvent evaporation method. The advantage over many other microencapsulation methods are rapid, convenient, easy to scale up, mild condition used, single-stage operations and close system (Burgess, 1994). Heat-sensitive substances can be coated by spray drying because exposure to elevated temperature is very short, normally ranging from 5 to 30 seconds (Gander, 1995). Moisture-sensitive materials can be encapsulated by using nonaqueous coating system. However, spray drying method causes a significant loss of product because of adhesion of microparticles to inside wall of spray dryer apparatus and can also generate agglomeration of microparticles.

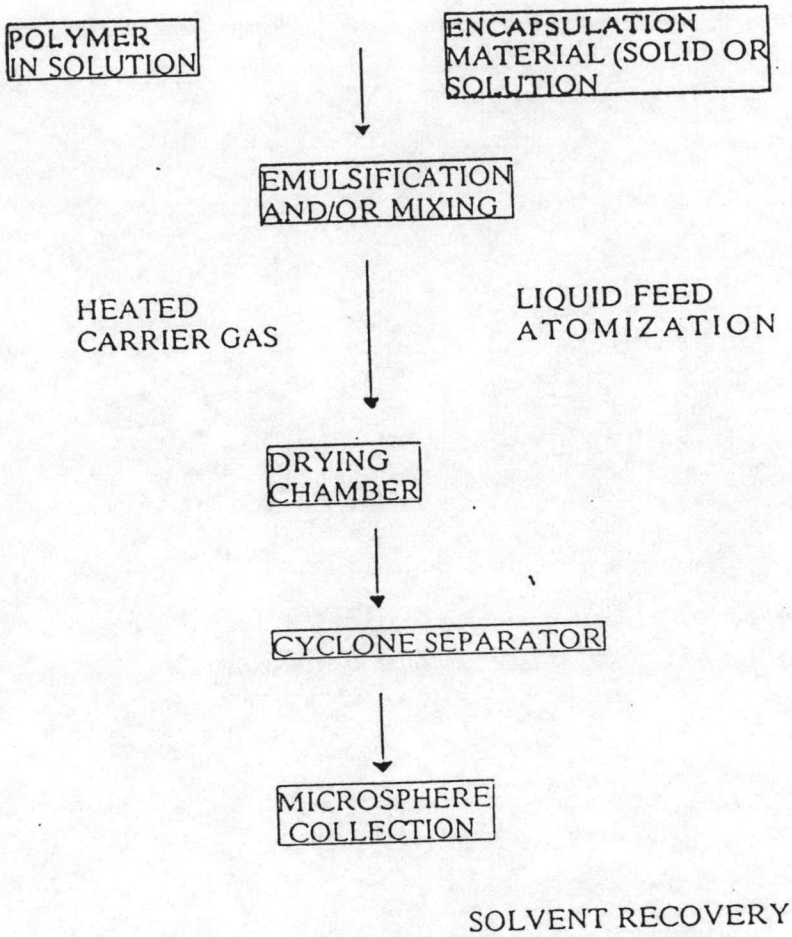


Figure 9 Flow diagram of spray - drying microparticle manufacturing method

3. Polymer-polymer incompatibility

This technology utilizes a polymer phase-separation phenomenon quite different from complex coacervation. Polymer-polymer incompatibility occurs because two chemically different polymers dissolved in a common solvent are incompatible and do not mix in solution. They essentially repel each other and form two distinct liquid phases. One phase is rich in polymer designed to act as capsule shell. The other is rich in the second, incompatible polymer. The incompatible polymer is present in the system to cause formation of two phases. It is not designed to be part of the final capsule shell, although small amounts may remain entrapped in the final capsule as an impurity. This type of encapsulation process generally does not involve any chemical reaction (Thies, 1996). The schematic diagram of encapsulation process based on polymer-polymer incompatibility illustrates in figure 10. The first step is to disperse core material in solution of polymer I. An incompatible polymer II solution in the same solvent is added to the system (hot system). This induces phase separation with formation of a polymer I - rich phase and a polymer 2 – rich phase. The solid core substance is dispersed in this two-phase system. Polymer I, more polar than polymer II, adsorbs preferentially on the surface of core material and causes a thin coating of shell mateial solution to engulf the particles of core material. When the system is cooled, the solid microparticles are formed and can be harvested. This process generally does not involve any chemical reaction. It normally is carried out in organic solvents and is used to encapsulate solids with a finite degree of water solubility. This technique is used to provide taste masking and prolonged drug delivery.

4. Interfacial polymerization

This technique involve the reaction of various monomers at the interface between two immiscible liquid phases to form a film of polymer that encapsulate the disperse phase. Generally, two reactive monomers are employed, one dissolved in the aqueous

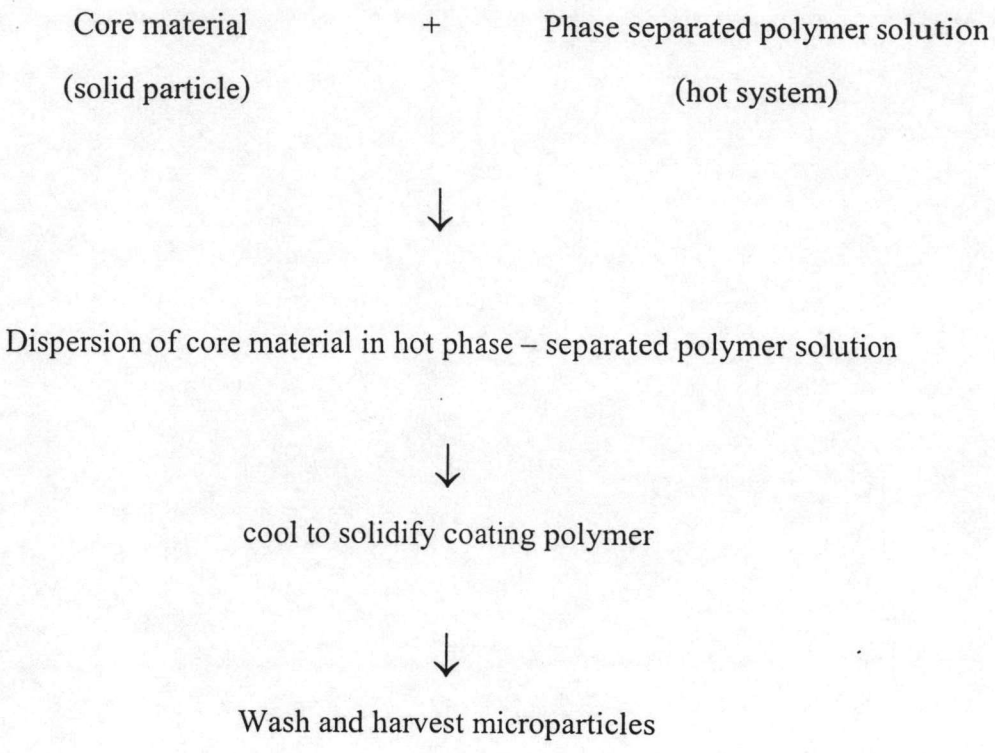


Figure 10 Schematic diagram of encapsulation process based on polymer – polymer incompatiblity

disperse phase containing a solution or dispersion of the core material, and the other dissolved after the emulsification step in the nonaqueous continuous phase (Kondo, 1979). A diagrametic representation of the process is depicted in figure 11. The monomers diffuse together and rapidly polymerize at the interface between the phases to form a thin coating and the by product of the reaction is neutralized by added material such as an alkaline buffer. The degree of polymerization can be controlled by the reactivity of monomer used, concentration, composition of either phase vehicle and temperature of the system (Deasy, 1984). The particle size of product can be controlled by the particle size of disperse phase. In the last 25 years many different monomer combination has been investigated for the microencapsulation of pharmaceuticals. One monomer is oil soluble and the other is water soluble as shown in Table 8 .

In the interfacial polymerization microencapsulation process, both dispersed and continuous phase serve as a source of a reactive polymeric species. These two kind of monomers used in this method are multifunctional monomers capable causing a polycondensation or a polyaddition reaction between them. This microencapsulation approach is able to encapsulate a wide range of core materials including aqueous solutions, water-immiscible liquids and solids.

Although this means is considerable interest, very few have been commercially exploited because of

1. toxicity problem associated with unreacted monomer, polymer or other constituents of system
2. excessive drug degradation caused by reaction with monomer
3. high permeability of coating formed to low molecular weight species including most drugs
4. fragility of microparticles formed
5. lack of biodegradability of products

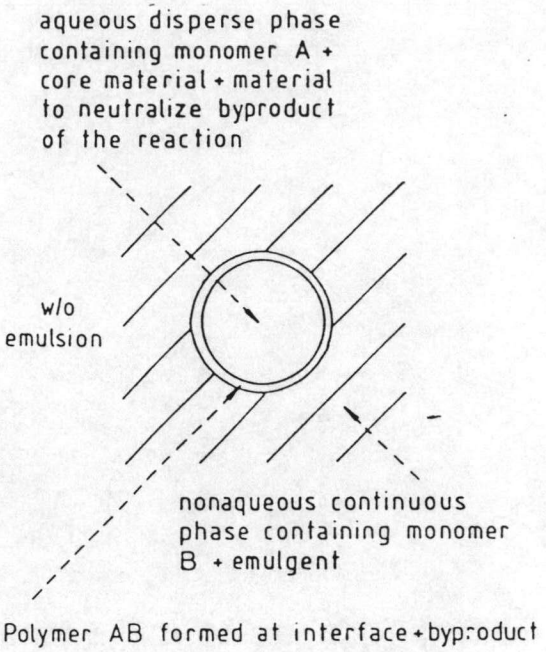


Figure 11 Schematic representation of microencapsulation of a droplet by interfacial polymerization

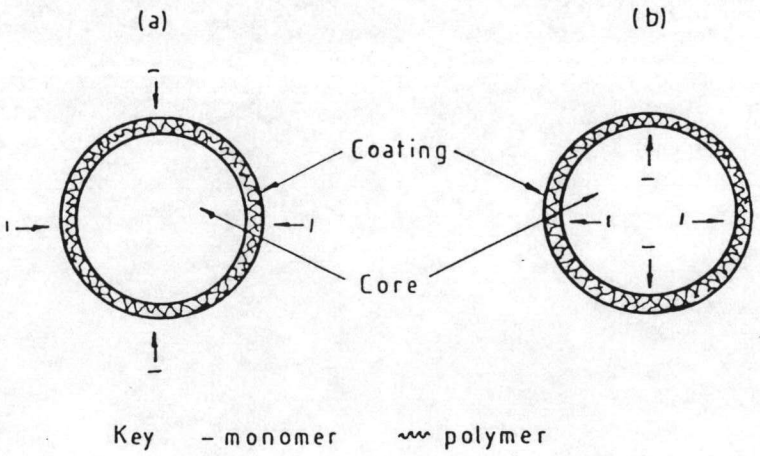


Figure 12 Schematic representation of in situ polymerization

Table 8 Principal monomer combinations for microencapsulation process by interfacial polymerization

Monomer in aqueous phase	Monomer in Nonaqueous phase	Polymer wall Material formed
Polyamine 1,6 –hexamethylene diamine Piperazine L – lysine	Polybasic acid halide Sebacoyl chloride Terephthaloyl chloride Terephthaloyl chloride	Polyamide Nylon 6-10 Polyterephthalamide Poly (terephthaloyl L-lysine)
Polyamine 1,6- hexamethylene diamine	Bis – haloformate 2,2-dichlorodiethyl ether	Polyurethane Polyurethane
Polyphenol 2,2- bis (4-hydroxy-phenyl) propane	Polybasic acid halide Sebacoyl chloride	Polyester Polyphenyl ester

5. In situ polymerization

In the in situ polymerization encapsulation, the monomer component and catalyst are fed exclusively from either the inside or outside of the core material drop. The reaction conditions are such that the monomer is soluble whilst the polymer is not. Thus, the polymerization occurs on surface of core material and the resulting insoluble polymer

coating forms around film covers the entire surface of core material (Kondo, 1979). The schematic representation of in situ polymerization is illustrated in figure 12 . The monomer used in this technique is liquid, water soluble, oil soluble or mixture of monomers. Furthermore, a low molecular weight polymer or precondensate can be used instead of monomer. Therefore, a variety of materials such as homopolymers, copolymers, graft copolymers and block copolymers are utilized as wall-constituting materials. The core material which may be liquid or solid must be insoluble in encapsulation medium. If core material is liquid, monomer and catalyst are present in either core material drops or in encapsulation medium. When core material is solid, polymer film-forming monomer and catalyst are resident in encapsulation medium. The medium for encapsulation may consist of water or organic solvent. In each system, the resulting polymer film should be insoluble in the medium. In order to encapsulate a water-insoluble solid powder or hydrophobic material, the encapsulation medium is water. The encapsulation process takes several hours to complete. Thus, a stable dispersion state of core material is achieved by adding surface active agent or thickener. Two mechanisms are involve in aqueous medium encapsulation. The first, lipophilic monomer is mixed with lipophilic core material and the second, source of water soluble monomer is from the medium. Here, either an aqueous solution or water sensitive material can be encapsulated. The organic solvent is used as polymerization medium. It is either immiscible or unreactive with water. Hydrophobic solid powder may be encapsulated by this approach. However, if a polymer nucleates at a point other than the surface of solid powder no capsule is obtained. Thus it is essential to place a catalyst on the surface of solid core substance.

However, the problem associated with unfavorable condition of polymerization such as use of high temperature for prolong periods and toxicity associated with residual monomers, catalysts and other manufacturing components have tended to limit the use of

in situ polymerization procedures for encapsulation of drugs and similar biological materials (Deasy, 1984).

6. Fluids under supercritical conditions

This technique involves placing a fluid at a temperature and pressure exceeding its critical point and using it as an extractant. The use of supercritical fluids as medium for the formation of microparticles for pharmaceutical application is developed. This means offer the important advantages over conventional microparticulate formation routes such as mildness of operating temperature and purity of product. Furthermore, it is a possible alternative to reduce health, environmental and safety risks from organic solvent (Frederiksen, 1997).

A supercritical fluid is any substance the temperature and pressure of which are simultaneously higher than the critical point values. It also means fluids in relatively narrow range of conditions $1 \leq T/Tc \leq 1.1$, $1 \leq P/Pc \leq 2$, where subscript c denotes critical point. Most of the changes in thermophysical properties associated with the transformation of a dilute gas into a dense fluid occur. Therefore, in supercritical region, thermophysical properties exhibit very high rate of change with respect to pressure and temperature (Dondeti, 1999). The critical points of same common supercritical solvents are listed in Table 9. Not all thermophysical properties, the most important property of a supercritical fluid is its large compressibility. All fluids are infinitely compressible at the critical point. A schematic density-pressure phase diagram of a pure fluid in the relative vicinity of the critical point is shown in figure 13. The critical isotherm ($Tr = T/Tc = 1$) has vertical slope at critical point which means that rate of change of density with respect to pressure is infinite. The portions of isotherms corresponding to liquid states have almost horizontal slopes because liquids have very small compressibilities. In the vicinity of critical point, then fluids are arbitrarily

compressible, small changes in pressure cause large changes in density. Thus, supercritical fluids are typically hundreds of times denser than gases at ambient conditions, but they are arbitrarily more compressible. Supercritical fluids can be almost liquid-like in density but their viscosities are intermediate between gas and liquid as shown in Table 10.

Table 9 Critical points of some common supercritical solvents

Substance	Critical temperature (° C)	Critical pressure (bar)
Nitrogen	-147	34
Carbon monoxide	-140	34
Argon	-123	48
Oxygen	-119	50
Methane	-83	46
Krypton	-64	55
Ethylene	9.3	50.4
Xenon	16	58
Fluoroform	26	48.8
Chlorotrifluoromethane	28.8	39.2
Carbon dioxide	31.1	73.8
Ethane	32.3	48.8
Sulfur hexafluoride	45.5	37.6
Propylene	92	45
Propane	96	42
Ammonia	132	111
Isobutane	134	36
Water	374.2	220.5

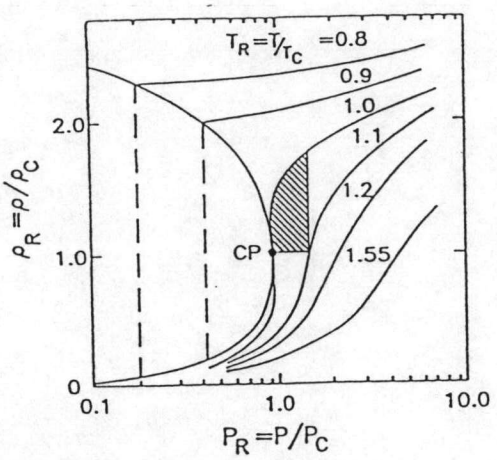


Figure 13 Schematic density - pressure phase diagram of a pure fluid

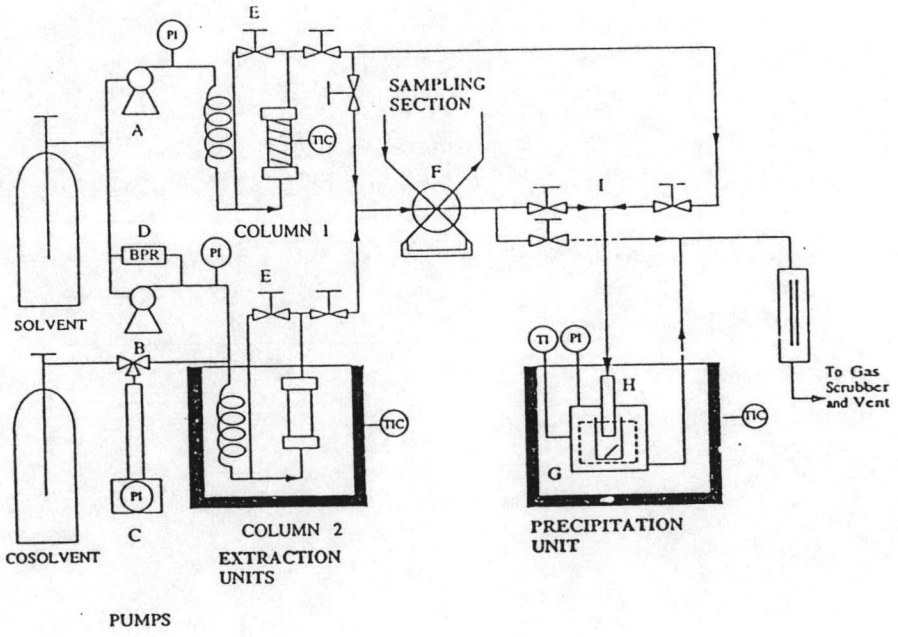


Figure 14 Typical schematic flow of RESS apparatus

Table 10 Physical properties of gases, liquids and supercritical fluids

	Density (kg/m^3)	Viscosity (Ns/m^2)	Diffusivity (cm^2/s)
Gases	1	10^{-5}	10^{-1}
Liquids	10^3	10^{-3}	10^{-5}
Supercritical fluids	700	10^{-4}	10^{-4}

Different thermophysical properties of supercritical fluids are used in different applications. For particle formation, the particles are produced with supercritical fluids by two ways : rapid expansion of supercritical solutions (RESS) and supercritical antisolvent processing (SAS) (Knutson, 1996).

6.1 Rapid expansion of supercritical solutions (RESS)

The solute is dissolved in a supercritical fluid at high pressure and precipitated by rapid decompression. A typical schematic flow of RESS apparatus is depicted in figure 14. The dissolution exploits the supercritical fluid's liquid-like solvent power as decompression and compressibility. The expansion is done by flowing the supercritical solution through a capillary, nozzle or calibrated restriction. Small pressure drops cause large density changes because of their large compressibility. These are accompanied by correspondingly large drops in solvent power; hence, the dissolved solute precipitates. However, expansion during nozzle or capillary flow is very fast, large supersaturation can be attained. Furthermore, pressure drop is a mechanical perturbation that travels at

speed of sound giving rise to uniform conditions within nucleating fluid. Thus, RESS is capable of producing very small and uniform particles. Furthermore, the solid product obtained is dry, solvent free and purify because the supercritical fluid is a dilute gas after expansion. RESS is an alternative to comminution for production ultrafine pharmaceutical powders especially for specialized applications such as pulmonary delivery system. However, the chief limitation of RESS is very low solvent power of common supercritical solvents toward potentially useful solutes such as proteins and high polymers.

6.2 Supercritical antisolvent technique (SAS)

SAS process uses low solvent power of supercritical fluids with moderate critical temperatures such as carbondioxide as an antisolvent. The solute is dissolved in a suitable liquid generally organic solvent. A supercritical fluid with a low affinity to solute and appreciable mutual solubility with organic solvent is added into liquid phase. The particle nucleation and growth from this system is governed by two fundamental processes. The first, an organic solvent expands by compressed antisolvent. The second, an organic solvent evaporates into antisolvent phase. The solvent expansion decreases solubility of precipitate within organic phase. Simultaneously, solvent evaporation increases concentration of solute within organic phase. The trace of organic solvent can be removed from resulting particulate phase by drying with pure supercritical fluid stream. Thus, SAS yields solvent-free particles in a single processing step. A typical diagram of SAS apparatus is illustrated in figure 15 . This technique has been applied to several monomeric and polymeric systems in pharmaceutical applications. It is used to form particles of proteins and many biological molecules.

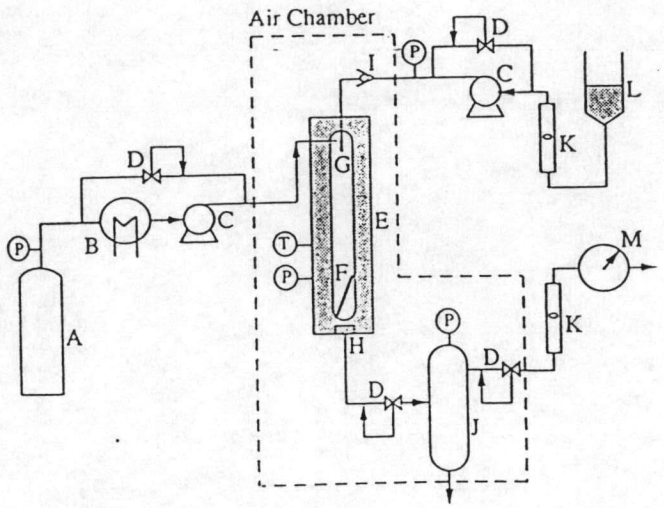


Figure 15 Schematic diagram of SAS apparatus : A) carbon dioxide , B) cooler , C) high - pressure pump , D) backpressure regulator , E) precipitator , F) glass sampler , G) nozzle , H) metal filter , I) check valve , J) depressurizing tank , K) rotameter , L) organic solution , M) dry test meter

7. Solvent evaporation and solvent extraction process

7.1 Solvent evaporation

This technique is based on evaporation of internal phase of an emulsion by agitation. This process can also be divided into

7.1.1 Single emulsion process

A) Aqueous emulsion (oil-in-water (o/w) emulsion)

The polymer is dissolved in a water immiscible, volatile organic solvent. The active substance to be encapsulated is either solubilized or suspended in the same medium to form solution or suspension. The entire mixture is then emulsified in an aqueous solution containing suitable emulsifier with appropriate stirring and temperature conditions to form o/w emulsion. The subsequent removal of organic solvent by evaporation harden oil droplets. The solid microparticles are obtained and then washed and collected by filtration or centrifugation. The microparticles are dried under appropriate conditions or are lyophilized to give final free-flowing product. A schematic diagram for this technique is depicted in figure 16.

The selection of dispersed and continuous phase is an important for successful microparticle formation. The dispersed phase must be immiscible or only slightly miscible with continuous phase and must have a boiling point lower than continuous phase. Dichloromethane (DCM) is widely used as continuous phase because of good solvent for polymers and high volatility and easily removed by evaporation. However, a potential toxicity of DCM, chlorinated solvent, is a major problem. It is considered as hazardous agent to environment and undesirable for use in manufacturing process. Other solvent such as ethyl aectate, methylethylketone have been used. The rate of solvent removal by evaporation technique affects characteristic of final microparticles obtained. The solvent evaporation rate depends upon temperature,

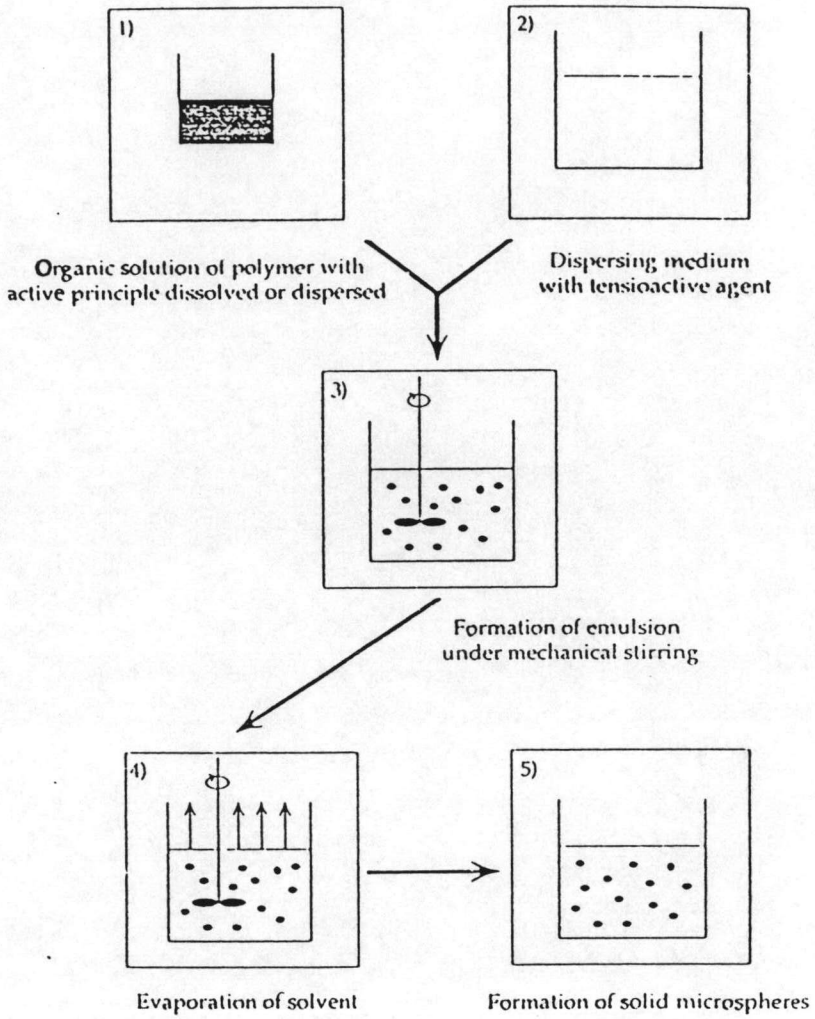


Figure 16 Schematic diagram of O / W emulsion technique

pressure and solubility parameters of polymer, solvent and dispersion medium. Very rapid solvent evaporation may cause local explosion inside droplets and lead to formation of porous structure on microparticle surface. The emulsifiers used in o/w solvent evaporation are hydrophilic polymeric colloids and/or anionic or nonionic surfactants. These include polyvinylalcohol (PVA), polyvinylpyrrolidone (PVP), alginate, gelatin, methylcellulose, hydroxyalkyl cellulose, hydroxypropylmethyl cellulose, Tweens, cetyltrimethyl ammonium bromide (CTAB) and fatty acid salts (Jeffery, 1991).

The emulsifier provides a thin protective layer around oil droplets and reduces coalescence and coagulation of droplets. The physicochemical properties and concentration of emulsifier significantly affect microparticle size, shape and encapsulation efficiency. Thus , a single or combination of emulsifier used in process is considerable to achieve necessary emulsifying action.

The o/w emulsification process is recommended to encapsulate lipid-soluble or low water soluble substances especially steroid hormones. Nevertheless, a major problem with this technique is poor encapsulation of moderately water soluble and water soluble components. These substances can diffuse out or partition from dispersed oil phase into aqueous continuous phase and microcrystalline fragments of hydrophilic materials are deposited on microparticle surface and dispersed in polymer matrix (Benita, 1984). The physicochemical characteristics of active ingredient such as partition coefficient, degree of ionization or surfactant character are an important role in localization of the two phases. The strategies to reduce the loss of water soluble active ingredients result in the increment of encapsulation yield. The solvation of medicinal substances in organic solution of polymers can be achieved by addition of hydrophilic cosolvents. The advantage of solubilizing active material is related to flexibility of

producing particles of extremely small sizes whose internal structures are more homogeneous irrespective of initial particle size. The water solubility of active ingredient can be reduced by chemically modifying prior incorporation in organic phase. This modification is simple to perform water soluble salt for commonly lipophilic substances. However, structural modification of drug may give toxicological problems. Another way to reduce leakage of active substance from oily droplets is modification dispersing phase of emulsion by saturating continous phase with active drug, adjusting pH of continuous phase or adding electrolyte to continuous phase. However, the validity and effectiveness of this strategy vary from case to case.

B) Nonaqueous emulsion (oil-in-oil (o/o) emulsion)

Oil-in-oil emulsification process is sometimes referred to as water-in-oil (w/o) emulsification process. It was developed to increase drug loading of water soluble drugs (Wang, 1996). A water-miscible organic solvent is used to solubilize drug in which polymers are also soluble. The solution is then dispersed into a continuous phase containing surfactant to yield o/o emulsion. The microparticles are finally obtained by evaporation of organic solvent from dispersed oil droplets, and are washed off by solvents such as n-hexane. The solvent used as dispersed phase includes acetonitrile, acetonitrile/water mixture, dichloromethane (DCM) and N,N- dimethyl formamide (DMF). The continuous phase consists of oils such as light mineral oil, silicone oil, liquid paraffin and cotton seed oil. An oil-soluble emulsifier such as Spans, Lecithin and polyoxyethylene fatty ethers (Brijs) has been used. However, this method used for encapsulation of a very limited number of active drugs including cytostatics, antiinflammatory, antimalarials and serum albumins.

7.1.2 Multiple emulsion process (water-in-oil-in-water (w/o/w) emulsion)

The active material to be encapsulated is added into an aqueous solution,

sometimes containing a viscosity building and/or stabilizing protein. The aqueous phase is added into an organic phase of polymer and is vigorously stirred to form primary microfine w/o emulsion. This emulsion is then emulsified in an external aqueous phase containing an emulsifier and gently stirred, so w/o/w multiple emulsion is formed. The organic phase acts as a barrier between two aqueous phase compartment preventing diffusion of active material toward external aqueous phase. The multiple emulsion is then subjected to solvent removal by evaporation process. The solid microparticles are obtained and then washed , collected by filtration or centrifugation and dried under appropriated conditions or lyophilized. A schematic diagram of w/o/w multiple emulsion is illustrated in figure 17. This technique is an efficient encapsulation of water soluble substances such as hormones, trophic factors, peptides, proteins and vaccines (Okada, 1994 and Brannon-Peppas, 1995). The encapsulation is much more effective when water solubility of drug is high (> 900 mg/ml) and partitioning between organic phase is disfavorable. The viscosity of water in oil primary emulsion is a crucial role in prevention of diffusion of active substance toward external aqueous phase. Therefore, the adding a viscosity enhancing agent such as gelatin, CTAB, PVA and etc., resulting in increase internal phase viscosity and hold active component in an aqueous internal phase. However, compatibility of active material may be considerable.

The size of microparticles decrease with decrease in internal aqueous phase volume and increase in shear rate to form inner emulsion. Further, an increase in stirring rate during emulsification of w/o emulsion into double w/o/w emulsion and increase in external aqueous phase volume lead to decrease particle size (Sah, 1995 and Igartua, 1997). The increment in encapsulation efficiency is obtained by increase in viscosity and volume of inner aqueous phase and increase in shear rate to form inner emulsion. Moreover, an increase in viscosity of whole w/o emulsion by decreasing volume of organic phase increases drug encapsulation and yields a dense core microparticle.

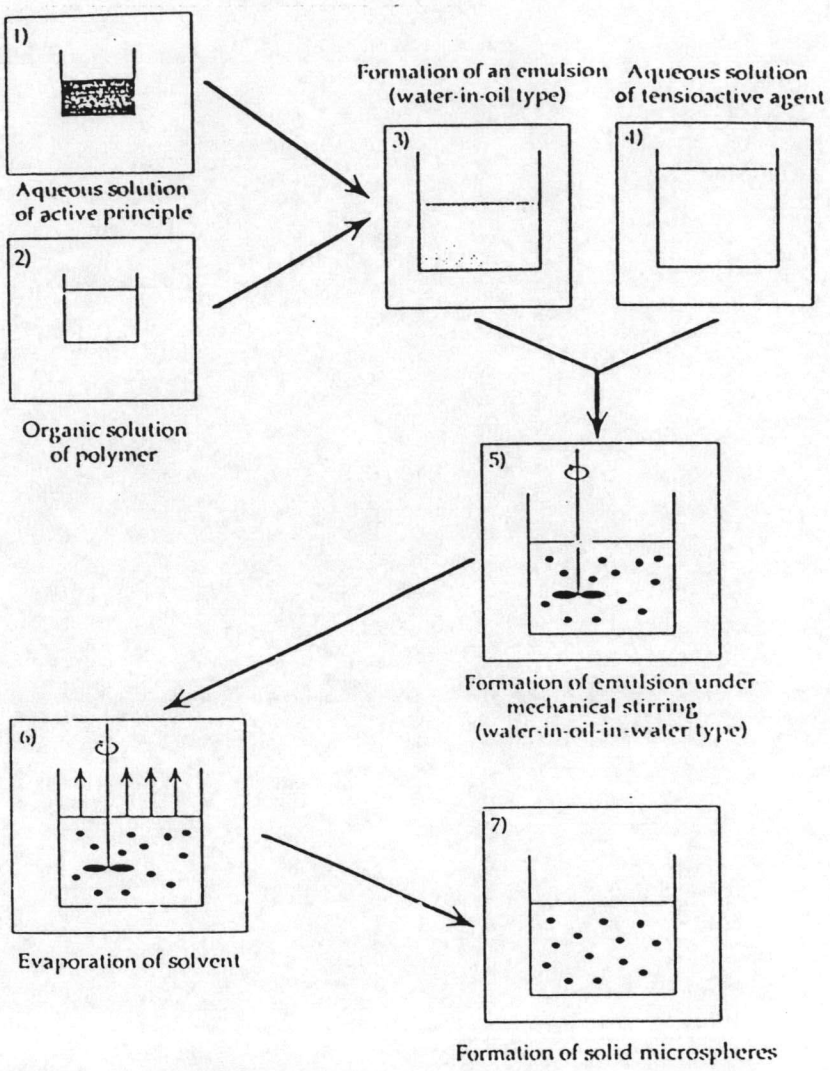


Figure 17 Schematic diagram of W/O/W multiple emulsion technique

Because high viscosity prevent migration of inner aqueous phase to outer aqueous phase due to local demulsification produced by vigorous stirring.

7.2 Solvent extraction

This approach is based on elimination of organic solvent of dispersed phase by diffusion of solvent in dispersing phase (McGee, 1994). It can be achieved by using large volumes of dispersing phase such as water or other quench medium with respect to dispersed phase or by choosing dispersed phase consisting of cosolvents, of which at least one has a great affinity for dispersing phase. Another one is used a dispersing phase which two solvents in which one acts as a solvent extractor of dispersed phase. A hybrid technology may also be envisaged where emulsification-evaporation is initialized then interrupted before volatile solvent is tolally eliminated. Thus, the native microparticles are transferred into a large volume of continuous phase where remaining solvent is eliminated by extraction. This means prevent the development of active drug crystals at microparticle surface during solvent evaporation stage or in continuous aqueous phase leading to decreased efficiencies in microencapsulation of drug. The transfer of embryonic microparticle in a large volume of water leads to rapid extraction of organic solvent which is found at periphery of dispersed globules which still contain some solvent. This leads to formation of a polymeric barrier at organic solvent continuous phase interface, which slows down diffusion of active drug from organic phase toward aqueous external phase and prevent crystal formation of active drug. The solvent extraction technique is faster than solvent evaporation technique. However, rate of solvent removal by extraction relies on temperature of water or other quench medium, ratio of emulsion volume to quench water/medium volume and solubility characteristics of polymer, solvent and dispersion medium (Yeh, 1995).

In solvent evaporation and solvent extraction process, however, many variables of process and formulation can affect the formation of microparticles. The factors such as nature and solubility of substances to be encapsulated, molecular weight, composition and concentration of polymers, active material/polymer ratio, organic solvent, mixing speed and temperature of emulsification process and volume and viscosity of dispersed and continuous phase have significantly affected microencapsulation process and microparticle properties.

Polymer materials

A controlled release delivery system is a crucial device in pharmaceutical applications. The rate, extent and duration of drug release in a predictable manner is regulated by physical properties of drug such as partitioning and diffusion or by progressive chemical erosion of polymer backbone. The physical and chemical properties of polymers can be systematically changed by repeating subunits. This versalility of polymers has led to design controlled drug delivery devices. They are used in matrix devices in which drug may be dissolved or dispersed and used as porous or nonporous membranes in reservoir devices. Further, they are increasingly being considered as carriers of drug molecules for controlled as well as site-specific delivery. Moreover, controlled release delivery devices may be administered via several routes including oral, transdermal, rectal, ocular and injectable.

The polymers used for drug delivery can be broadly classified into several groups which may be defined as follow.

1. Nonbiodegradable polymers

These polymers are inert and do not underg any chemical change in vivo, so they are eliminated or extracted intact from site of administration. They act as a rate-limiting barrier to transport and release of drug from devices. These polymers include silicones, polyethylene vinylacetate (EVA), polydimethyl siloxane (PDS), polyether urethane (PEU), ethylcellulose (EC), cellulose acetate (CA), polyethylene (PE) and polyvinyl chloride (PVC).

The drug release from most matrix devices that use nonbiodegradable polymers is primarily by a solution-diffusion mechanism. However, other mechanisms involve in drug release from devices made by these polymers. The partitioning of drug between polymer and water affects drug release kinetics (Thombre, 1990). In vivo situation, generally, the external hydrodynamic conditions (maintenance of sink conditions) may be extremely crucial in governing rate of drug release. However, synthetic hydrophobic polymers such as PE, PVC and copolymer systems have been primarily limited use in controlled release delivery system due to their low permeability to drug substances.

2. Water-soluble polymer

These polymers are the most extensive use as drug delivery matrices. They contain hydroxyl, ethyleneoxy, amine and carboxylic acid groups in their structures. They dissolve in the body as a result of hydration, ionization or protonation with respect to their functional groups . Some of the more widely used water soluble polymers are listed in Table 11.

Although they are water soluble, their rates of dissolution vary over a wide range. The rate of water dissolution can be changed rather significantly by changing

Table 11 Examples of water soluble polymers used in drug delivery

Polyethylene glycol	Dextran
Poly(vinyl alcohol)	Sodium alginate
Poly(vinyl pyrrolidone)	Poly(acrylic acid)
Poly(2-hydroxyethyl methacrylate)	Poly(methacrylic acid)
Poly(acrylamide)	Poly(maleic acid half esters)
Hydroxypropyl cellulose	Poly(sodium styrene sulfonate)
Hydroxypropylmethyl cellulose	Poly(dimethylaminoethyl methacrylate)
Sodium carboxymethyl cellulose	Poly(vinyl pryidine)
Gelatin	Cellulose acetate *N,N,*-diethylaminoacetate
Starch	

Table 12 Examples of biodegradable polymers used in drug delivery

Category I

Poly(maleic anhydride copolymers)

Category II

Gelatin-formaldehyde
Acrylamide - *N, N'*-methylenebisacrylamide
N-vinyl pyrrolidone - *N, N'*-methylenebisacrylamide
Fumaric acid/polyethylene glycol - *N*-vinyl pyrrolidone
Fumaric acid/diglycolic acid - *N*-vinyl pyrrolidone
Fumaric acid/ketomalonic acid - *N*-vinyl pyrrolidone
Fumaric acid/ketoglutaric acid - *N*-vinyl pyrrolidone

Category III

Polylactic acid	Polyanhydrides
Polyglycolic acid	Polyorthoesters
Polycaprolactone	Poly(amino acids)
Polyhydroxybutyrate	Psuedopolyamino acids
Polyhydroxyvalerate	Polyphosphazenes

polymer molecular weight, with increase in molecular weight resulting in decrease dissolution rate.

Water soluble polymers have a variety of pharmaceutical applications. Water soluble drugs are released from a polymeric device by a diffusive mechanism whereas drugs with poor aqueous solubility are released by an erosion mechanism corresponding to slow dissolution of polymer. However, these polymers can be used alone or in combination with hydrophobic polymers to provide devices that slowly release over time.

3. Hydrogels

Hydrogels are crosslinked of water soluble polymers by nondegradable or nonhydrolyzable linkages. These polymers are nonbiodegradable and nonsoluble in the body but they do absorb large quantities of water and swell (Dunn, 1991). Common examples include polyhydroxyethyl methylacrylate (pHEMA), cross-linked polyethyleneoxide, cross-linked polyvinylalcohol, cross-linked polyvinylpyrrolidone, polyacrylamide. Hydrogels are inert, removed intact from site of administration and function by forming a rate-limiting barrier to transport and release of drugs. Further, hydrogels are highly permeable to drug molecules due to plasticizing effect of water on polymer. Synthetic hydrogels are compatible with body fluids and have been employed as controlled release system. Their biocompatibility is due to their ability to simulate natural tissue interface because of their high water content and special surface properties. The kinetic of drug release from hydrogel matrix systems is complicated by presence of a swelling front that moves inward. The dimension of device constantly change over time and diffusivity of species are different in swollen and nonswollen region (Thombre, 1990). A mathematical analysis is complicated by concentration-dependent diffusion coefficients.

The hydrogels provide a restrict aqueous environment for diffusional migration of macromolecular drug. Jhon and et al (1973) and Zentner (1979) suggested that for small molecules, a distinction be made between transport through a domain composed of bulk water (pore mechanism). and a domain composed of polymer segments, interfacial water and bound water (partition mechanism). The pore mechanism is dominant for hydrophilic macromolecules. The first application of hydrogel to controlled delivery of macromolecules was found in 1972 by Davis. His work included controlled delivery of IgG, leutinizing hormone, bovine serum albumin, prostaglandin F_2 and sodium iodide by using polyacrylamide and polyvinylpyrrolidone gels crosslink with 20% w/w N, N′ – methylenebisacrylamide. Sato and Kim (1984) described the use of hydrogels of hydroxyethyl methacrylate, methoxyethyl methacrylate and methoyethoxyethyl methacrylate as membranes for diffusion-controlled delivery of water soluble solutes with a wide range of molecular weights such as sodium acetate, glucose, maltose, insulin, cytochrome C and albumin. The diffusivity depends on solute size and membrane hydration. The model of permeation involves both pore and partition mechanisms. Sorensen and Peppas (1997) illustrated that change in permeability of crosslinked polyvinylalcohol to serum albumin, insulin and myoglobin was achieved by systematically varying in distance between crossliking. The greater size of protein, the greater sensitivity of diffusion coefficient to change in crosslinking density was observed. They concluded that the crosslink density of hydrogels had been used as a means of controlling both degree of hydration and permeability of hydrogels to proteins.

Heller et al. (1983) prepared biodegradable hydrogels as shown in figure 18. The first polymer was prepared from polyethylene glycol and fumaric acid and then crosslinked by copolymerization with N-vinylpyrrolidone. Then, a structurally similar

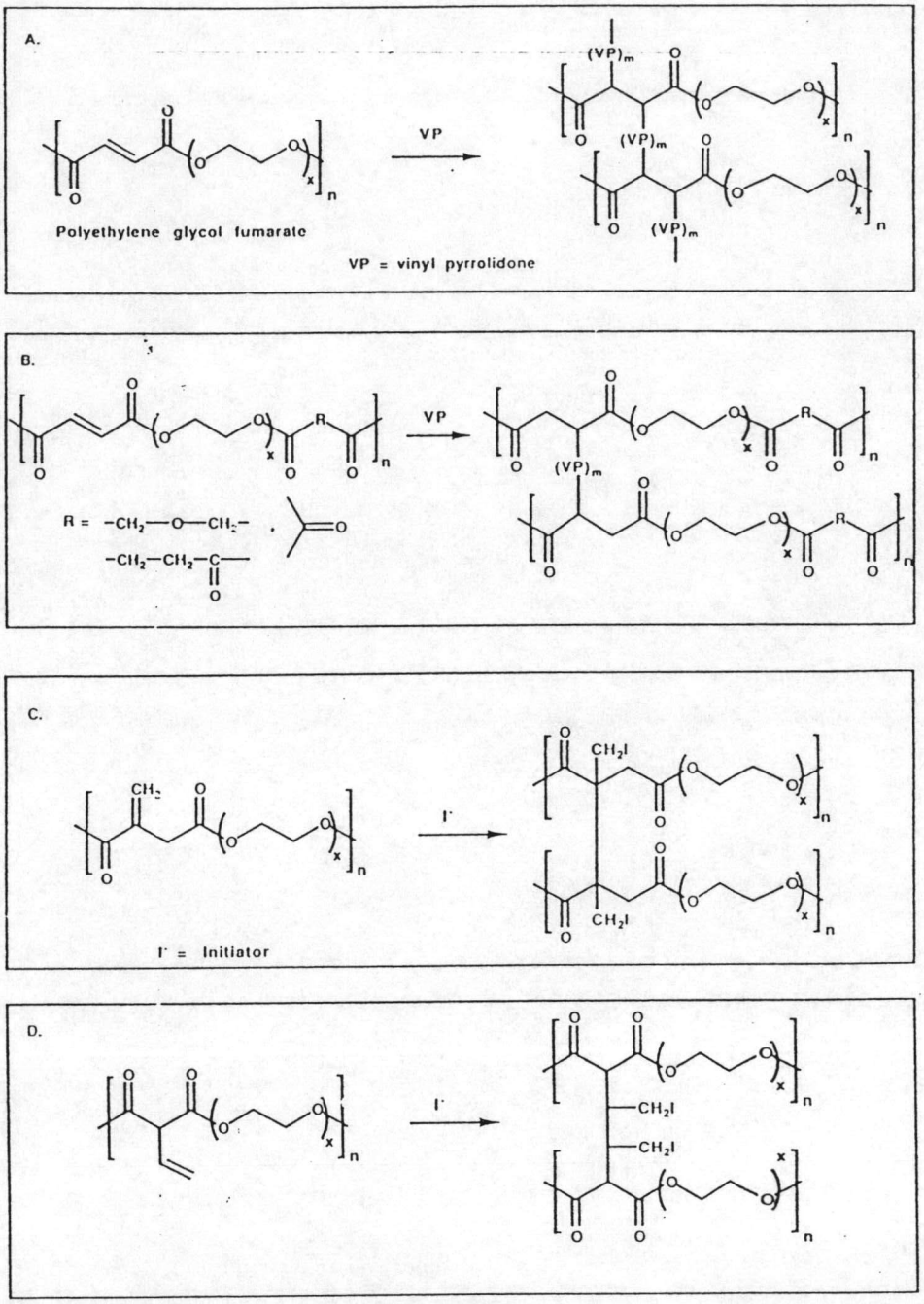


Figure 18 Preparation of biodegradable hydrogels : A) esters of polyethylene glycol and fumaric acid, crosslinked with N-vinylpyrrolidone , B) esters of polyethylene glycol, fumaric acid, and either ketomalonic, diglycolic or ketoglutaric acid, crosslinked with N- vinyl- pyrrolidone , C) esters of polyethylene glycol and itaconic acid, radical crosslinked and D) esters of polyethylene glycol and allylmalonic acid, radical crosslinked

but more rapidly hydrolysed hydrogel was prepared from combination of fumaric acid with ketomalonic acid, 1:1 ketoglutaric acid or 1:1 diglycolic acid. These structure are more hydrolytically labile because of presence of electron withdrawing groups proximate to ester function. The drug released from these gels as chain scission resulted in slow dissolution. The zero-order kinetic was observed. However, erosion of these hydrogels produced water soluble but nondegradable polymer, PVP as a by-product. The other group of hydrogels which totally degradable to low molecular weight fragments was prepared by vinyl polymerization of polyesters of itaconic and allymalonic acid. The release rate from these hydrogels was controlled by chemical structure and concentration of unsaturated ester in solution prior to gelation. In addition, crosslinking of hydrogels can be achieved by both physical and chemical means such as by association of crystalline of hydrophobic polymer blocks.

4. Biodegradable polymers

These polymers as denote as water-insoluble polymers which by means of a chemical reaction in the body are converted slowly to water soluble materials. Several methods for achieving this change in the body are described. First, polymer can have a side chain substituent which undergoes hydrolysis in the body to produce hydroxyl, carboxyl or other hydrating groups that act to make the entire polymer water soluble. The second approach is to crosslink a water-soluble polymer with a hydrolyzable crosslinking agent resulting in insoluble polymer. The crosslinking group is hydrolyzed or degraded in the body to give a water soluble polymer. The third and more frequently used technique is to use water insoluble polymers that contain hydrolyzable functional groups directly in the polymer chain. When polymers are hydrolyzed, the chain is slowly reduced to shorter and shorter chain segments which eventually become water soluble. The main advantage of these polymers is that extremely high molecular weight polymer with good mechanical properties can be used and subsequently eliminated from

the body when polymer chain has been reduced to water soluble fragments. The examples of these three categories of biodegradable polymers are listed in Table 12.

However, more attention has been devoted to the third category of biodegradable polymers. The polymers in this group include polylactic acid (PLA), polyglycolic acid (PGA), copolymers of lactic acid and glycolic acid (PLGA) polycaprolactone (PCL), poly (hydroxybutyrate) (PHB), poly (ortho-esters), polyanhydrides and polyphosphazenes. Biodegradable polymers have many important applications in medicine such as absorbable sutures, surgical implants and controlled drug delivery devices.

4.1 Lactide/glycolide polymers and copolymers

Polylactic acid (PLA), polyglycolic acid (PGA) and copolymers of lactic and glycolic acid (PLGA) have been used in a variety of medical applications including controlled drug delivery system for a long time. They are excellent biocompatibility and biodegradability, little or no local and systemic toxicity, no need for removal of device from body, easy to formulate into drug delivery devices and approved by United States Food and Drug Administration (USFDA) that generated immense interest in these polymers (Dunn, 1991).

α - hydroxypropionic acid or lactic acid has an asymmetric carbon atom (C *) that confers optimal rotary ability on molecule giving two optical isomer D(-) lactic acid and L(+) lactic acid. If water is removed by vacuum distillation, optically active or racemic lactic acid can form six-membered cyclic dimers giving an optically acitive D (-) lactide, L(+) lactide and racemic DL-lactide as shown in figure 19. The racemic DL-lactide consists of a mixture of D(-) lactide and L(+) lactide. It is also an optically inactive meso-lactide. PLA can be prepared by heating with an acidic catalyst such as

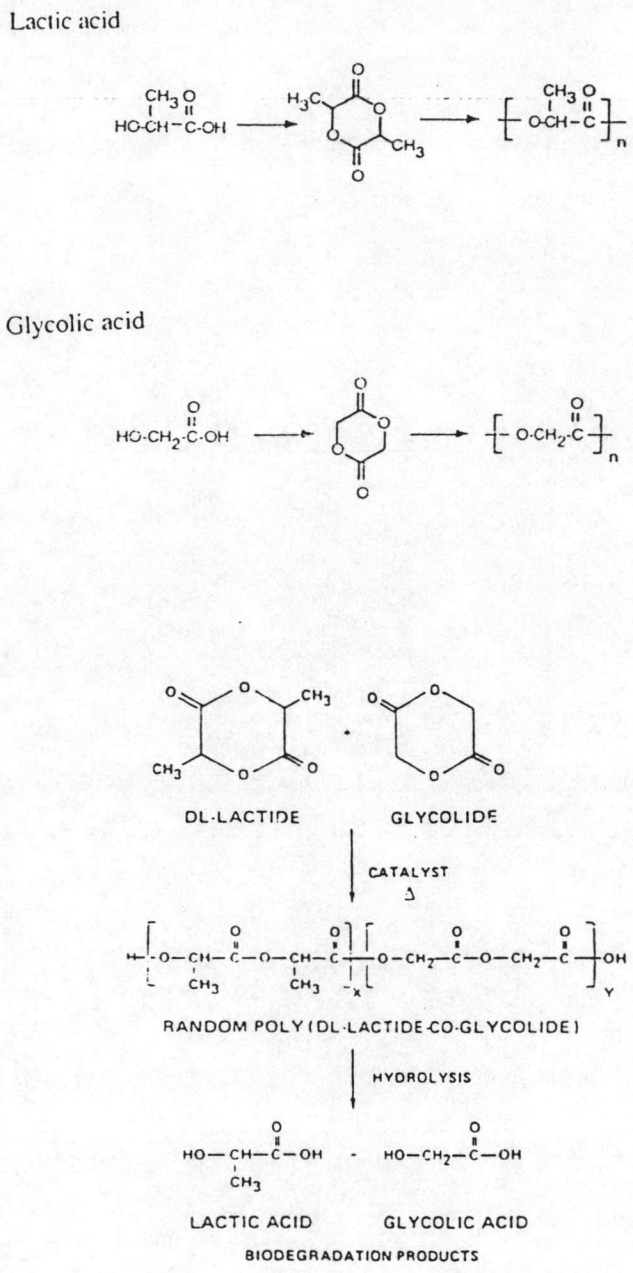


Figure 19 Synthesis, structure and biodegradation of PL / PG and copolymers

$SnCl_4$. Therefore, PLA can be an optically active stereoregular polymer poly (D-lactic acid, D-PLA), poly (L-lactic acid, L-PLA) and an optically inactive racemic polymer poly (DL-lactic acid, DL-PLA). The polymer made of optically pure isomers are tough, inelastic and highly crystalling and do not readily hydrolyse because of high regularity of polymer chain structure (Brophy, 1990). They have low permeability and slowly degrade over several months. Whereas racemic form is glassy, completely noncrystalline or amorphous due to irregularities in its polymer chain structure. So it is more permeable and more rapidly hydrolysed. L-isomer is easily metabolized by humans but D-isomer is not, so D-PLA is rarely used for drug delivery systems. Further, racemic DL-PLA is most frequently used for controlled drug delivery systems preferred than L-PLA because DL-PLA enables more homogeneous dispersion of drug in polymer matrix. PLA degrades by hydrolysis of ester bonds in bulk polymer. The degradation rate is depend on polymer molecular weight.

α - hydroxyacetic acid or glycolic acid is no asymmetric carbon atom. It has not optically active property. The dimer of glycolic acid is glycolide, which exist in two isomeric configuration, α - and β- form. The α - crystalline form is more stable than β -form. Thus, α - form is preferred to prepare of high molecular weight polymer. The general structure of glycolic acid and glycolide is shown in figure 19. PGA can be synthesized by heating with an acidic catalyst. It is glassy, brittle and highly crystalline because it lacks methyl side group of PLA (Jain, 1998). PGA hydrolyzes very slowly so it has seldom been used for drug delivery (Smith, 1990).

A wide range of PLGA copolymers can be developed. Low molecular weight PLGA can be prepared by direct condensation (polyesterification) of lactic and/or glycolic acid. This method yields PLGA having molecular weight around 10,000. Intermediate and high molecular weight PLGA can be prepared by ring – opening

polymerization of cyclic dimers (cyclic diester of lactic and/or glycolic acid) as starting materials. The polymers synthesized from this method have molecular weight ranging from 10,000-40,000. The low molecular weight PLGA has limited in biomedical application because of its poor mechanical strength and faster degradation. In general, PLGA copolymers form glassy structure with lower crystallinity that have various hydrolysis rate controllable by composition of lactic and glycolic acid. The glass transition temperature (Tg) of PLGA copolymers is above physiological temperature. Hence, they have a fairly rigid chain structure which gives them significant mechanical stregth to be formulated as drug delivery devices. Tg of PLGA copolymers decreases with a decrease of lactide content and with a decrease in molecular weight of PLGA. The crystallinity of PLGA depends upon type and molar ratio of individual monomer components, lactide and glycolide, in copolymer chain (Jain, 1998). Lactide-rich PLGA copolymer are less hydrophilic, absorb less water and subsequently degrade more slowly because lactic acid is more hydrophobic than glycolic acid. PLGA containing 50:50 ratio of lactic and glycolic acid are hydrolyzed much faster than those containing higher proportion of either of two monomers. PLGA prepared from L-PLA and PGA are crystalline copolymers whereas from DL-PLA and PGA are amorphous. PLGA copolymers containing glycolide less than 70% are amorphous. However, degree of crystallinity and melting point of polymers are directly related to molecular weight of polymer.

The degree of crystallinity of PLGA directly influences in mechanical strength, swelling behavior, capacity to undergo hydrolysis and biodegradation rate. Generally, the more amorphous a polymer is, the more permeable it is. The crystallinity gives strength and brittleness of polymers but reduces permeability. In addition, the molecular weight and polydispersity index of polymers are very important factors that affect mechanical strength of polymer and ability to be formulated as drug delivery devices.

Moreover, these properties may also control hydrolysis and biodegradation rate of polymer. In vivo half-life of low molecular weight polymers are shorter than high molecular weight polymers. Further, other factors such as copolymer composition and geometric regularity of individual chains significantly affect mechanical strength of polymers.

PLGA undergoes degradation in an aqueous environment as hydrolytic biodegradation through cleavage of backbone ester linkage both in vitro and in vivo followed a first order rate. The polymer chains undergo bulk degradation occurs at a uniform rate. PLGA biodegradation occurs through random hydrolytic chain scission of swollen polymers. The carboxylic end groups present in PLGA chain increase in number during biodegradation process as individval polymer chains are cleaved. The end group are known to catalyse degradation process. The biodegradation rate of PLGA copolymers is dependent on molar ratio of monomers, copolymer composition, degree of crystallinity, molecular weight and glass transition temperature. Surface erosion does not significantly contribute to degradation process. However, the role of enzymes in PLGA degradation is unclear. Most publications indicated that PLGA degradation is purely through hydrolysis, not involve enzymatic reaction but some suggested an enzymatic role in PLGA breakdown.

The biodegrade products of PLGA polymers are lactic and glycolic acid, which are natural metabolites. Lactic acid enters tricarboxylic acid cycle and is metabolized and subsequently eliminated from body as carbondioxide and water. Glycolic acid is either excreted unchanged in kidney or enters tricarboxylic acid cycle and is eventually eliminated as carbondioxide and water (Jalil, 1990).

A wide range of drugs such as narcotic antagonists, contraceptive steroids, chemotherapcutic agents, local anesthetics, antimalarials, antibiotics, peptides and proteins, vaccines and etc. have been reported using this group of polymer to form implants, pseudolatices, ocular devices, microparticles and nanoparticles as drug delivery devices (Lewis, 1990).

4.2 Polycaprolactones

Poly (ε - caprolactone) (PCL) is a linear polyester which has a general structure as shown in figure 20. PCLs have been synthesized from anionic, cationic or coordination polymerization of ε - caprolactone. A schematic description of caprolactone polymerization using three types of initiators is shown in figure 21.

PCL is soluble in chlorinated and aromatic hydrocarbons, cyclohexanone and 2-nitropropane. It is in soluble in aliphatic hydrocarbons, diethyl ether and alcohols. PCL homopolymer is highly crystalline, melts at 59-64 $^{\circ}$ C with a Tg of –60 $^{\circ}$ C , so it is a rubbery polymer at physiological temperature. Copolymerization with lactide increases Tg with increase in lactide content in copolymer. The crystallinity of polymer decreases with increase in polymer molecular weight.

PCL and its copolymer degrade both in vitro and in vivo by ester group hydrolysis by random chain scission process (bulk hydrolysis). Both in vitro and in vivo degradation follow a first order rate. The degradation rate is dependent upon size and shape of device and additives. The polymer degrades in two phases. The first phase, a random hydrolytic chain scission occurs that result in a reduction of polymer molecular weight. The second phase, low molecular fragments and small polymer particles are carried away from site of administration by solubilization in body fluids or by phagocytosis which result in a weight loss. The polymer degradation occurs at a slow

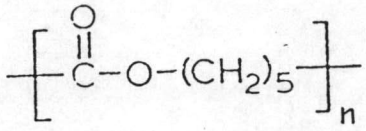
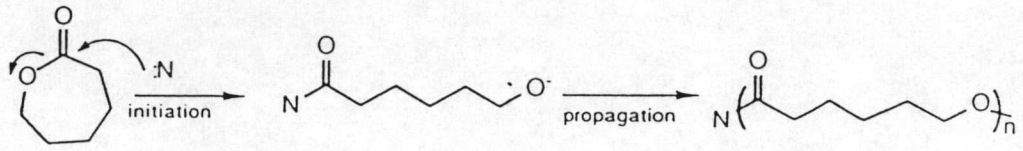
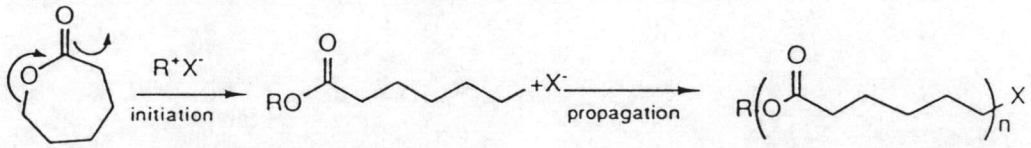


Figure 20 Structure of polycaprolactones

(A) Anionic:



(B) Cationic:



(C) Coordination:

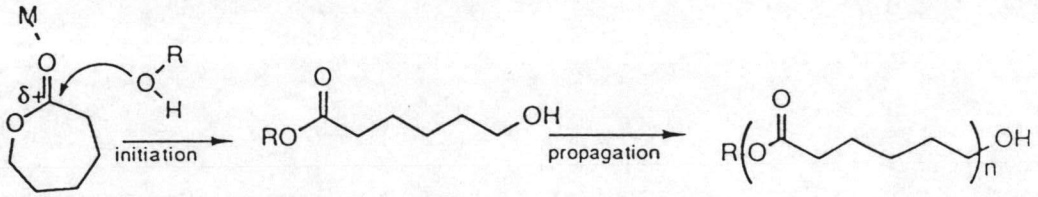


Figure 21 Polymerization of ε-caprolactone using (A) anionic, (B) cationic, and (C) coordination catalysts.

rate. Complete degradation and elimination of PCL homopolymer may last for 2-4 years. Copolymerization with lactide and glycolide significantly increase degradation rate because copolymer has much lower crystallinity than homopolymer. The degradation rate of copolymer of PCL and PLA increases in direct proportion to PLA content between ranging from 11% to 90% PLA . Moreover, degradation rate can be increased by addition of oleic acid or tertiary amines into polymer that catalyses chain hydrolysis.

PCL is used as drug delivery devices because of its biocompatibility, biodegradability, minimal tissue reaction, lack of inflammatory response, non-mutagenic effect and nontoxicity (Pitt, 1990). However, drug delivery research with PCL has been largely confined to low molecular weight substances especially contraceptive steroids and narcotic antagonists. In addition, the high permeability of PCL to therapeutically active macromolecules renders it a suitable excipient for use in long term delivery of proteins and peptides (Huatan, 1995).

4.3 Poly (β- hydroxybutyrate) and copolymers

Polyhydroxybutyrate (PHB) as shown in figure 22. is a polymer that has received attention as a biodegradable material in medical applications as well as drug delivery devices. Large quantities of PHB of accurately defined molecular weight can be produced from D (-) – hydroxybutyric acid by a variety of bacteria. However, low molecular weight polymer can synthetically be prepared. In commercial, PHB is prepared via a biosynthetic route using certain strains of bacteria.

PHB is a linear polyester with isotactic, brittle, optically active and existing in a regular helical conformation. It has been shown to be highly crystalline thermoplastic polymer with a melting point of around 180 ^{o}C and glass transition

temperature between 20 °C and 30°C. An electron micrograph reveals rectangular shaped crystals. Copolymers of PHB with poly (3-hydroxyvalerate) (PHV) have been used in drug delivery devices. PHV reduces crystallinity and melting point of PHB homopolymer and improves processibility. The 1:1 copolymer with 3-HV melts at 91 °C (Domb, 1994).

PHB exhibits a solubility aging phenomenon (Brophy, 1990). Freshly isolated polymer is soluble in either chloroform or methylene chloride, the solubility is lost after several weeks.

PHB is intrinsically unstable at elevated temperatures due to effects of hydrolysis and β - elimination reactions. Just above melting point, temperature ranging from 170 °C to 200 °C , changes in molecular weight occur in polymer. Two mechanisms involve a molecular weight change are shown in figure 23. First, a random chain scission at ester groups to give carboxyl and vinyl groups and an overall reduction in polymer molecular weight. Second, esterification occurs with condensation between terminal hydroxyl groups present in original polymer and increasing concentration of carboxyl groups formed in chain scission process, which delays or slows down initially overall reduction in molecular weight.

PHB, as well as PGA and PLA, has been as a potentially useful biodegradable drug carrier. It is biodegradation by enzymatic hydrolysis. Copolymers with a higher fraction of 3-HV and low molecular weight polymers are more susceptible to hydrolysis. The degradation product is 3-hydroxybutyric acid which found as endogeneous substance. Further, it is compatible with living cell with no abnormal inflammatory reactions (Thomber, 1990). The rate and duration of drug release from PHB devices depend on drug loading. The principle mechanism of drug release is diffusion of drug

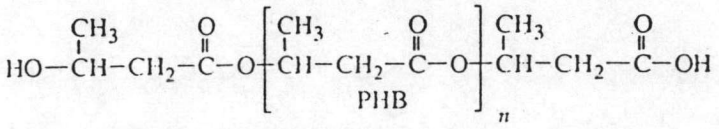
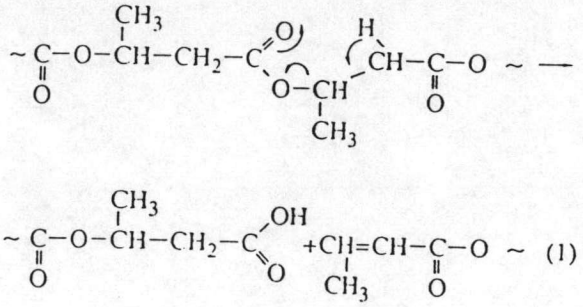


Figure 22 Structure of polyhydroxybutyrate

Chain Scission



Esterification

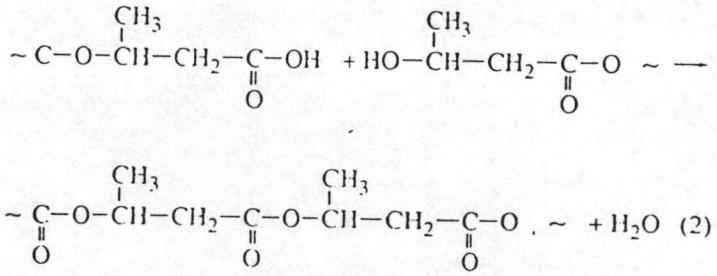


Figure 23 The mechanisms involve in molecular weight change of PHB

rather than erosion of polymer matrix. The duration of drug release varies from a day to around 50 days. The duration of release can be shortened by adding additive such as fatty acids and fatty acid esters. The release mechanism in this system is complex. However, the applications of PHB in controlled release delivery system reported to date are minimal.

4.4 Poly (ortho esters)

The general synthesis of the first family of poly (ortho esters) is prepared By transesterification of a cyclic ortho-ester with a diol. The molecular weight of polymers significantly depend upon type of diol and catalyst used for synthesis. They contain acid-sensitive ortho ester linkage in polymer backbone. Poly (ortho esters) are specially designed for use in a controlled-delivery system in which drug is fully immobilized and polymer erosion is limited to outer surface of solid device (Pitt, 1990). The drug releases by surface erosion with minimal contribution from diffusion. The release rate is proportion to initial drug load and external area of device. The duration of release is proportion to thickness of device.

Poly (ortho esters) are hydrophobic in nature and relatively stable under alkaline or neutral conditions but readily hydrolyze in acidic media. Hydrolysis of this polymer is both general and specific acid catalyzed. The degradation products from hydrolysis are mainly cyclohexanedimethanol and ν - butyrolactone which rapidly hydrolyse to 4-hydroxybutyric acid. The 4-hydroxybutyric acid further catalyses breakdown of orthoester linkage leading to bulk erosion of matrix. The erosion of polymer and drug release from polymer matrix is strongly influenced by acidity of surrounding medium or eroding interface. Therefore, a variety of erosion rate and drug release duration can be obtained by incorporating substances, that influence pH at eroding interface, in matrix (Heller, 1990). Incorporation with a water – soluble basic

salt in polymer matrix can imbibe water by an osmotic mechanism and swells. The polymer is stabilized and erosion to surface layer do not take place. The release is zero-order due to a swelling-controlled release. Basic or mildly acidic salts with low water solubility such as calcium lactate or magnesium hydroxide can add in delivery device that release active agent at a constant rate for a period of a year. Furthermore, the acid catalyzed degradation has been effectively utilized to accelerate erosion rate by incorporating an acid producing agents such as acid anhydride into polymer matrix. The pKa and amount of acid anhydride mixed in polymer matrix offer a convenient method to tailor erosion rate and release duration of matrix devices. The duration of release from such devices ranges from hours to weeks.

This polymer can be crosslinked using monomer such as ketene acetal that has a functionality greater than two. The gereral method used for synthesis crosslinked polymer is by reacting prepolymer with triols or a mixture of diols and triols. The crosslinking polymer is amenable to incorporation of proteins and peptides (Domb, 1994).

4.5 Polyanhydrides

Polyanhydride is a one class of biodegradable polymer under development as carrier for bioactive molecules including proteins and peptides. This polymer is biocompatible with body fluids and tissues nonmutagenic, nonteratogenic, noninflammatory and nontoxic (Chasin, 1990). Two different groups of polyanhydrides are synthesized. One group is aromatic polyanhydride which synthesized in 1909 and another group is aliphatic polyanhydride which synthesized in 1930. Polyanhydrides are prepared by condensing polyacids. Schematic diagram of polyanhydride synthesis is shown in figure 24.

Almost all polyanhydrides show some degree of crystallinity. The homopolymer such as sebacic acid (SA), bis (carboxyphenoxy) propane (CPP), bis (carboxyphenoxy) hexane (CPH), and fumaric acid are highly crystalline and crystallinity of copolymer in most cases by monomer of highest concentration are obtained. Copolymers with a composition close to 1:1 are essentially amorphous. The melting point of aromatic polyanhydrides is much higher than that of aliphatic polyanhydrides. The melting point of aliphatic-aromatic copolyanhydrides is proportional to their aromatic content. Polyanhydrides dissolve in solvents such as dichloromethane and chloroform. However, aromatic polyanhydrides display much lower solubility than aliphatic polymer. Copolymers of two different aromatic monomers improve solubility and decrease melting point.

Polyanhydrides are capable of undergoing hydrolysis process. Anhydride linkage is extremely susceptible to hydrolysis in presence of moisture to generate dicarboxylic acids. Hydrolysis of monomeric anhydrides is catalysed by both acid and base. The hydrolytic degradation rate increases with increasing pH. However, the degradation rate enhanced by incorporating aliphatic monomer such as sebacic acid into polymer and slowed by increasing methylene group into backbone of polymer to increase hydrophobicity (Heller, 1994).

Hydrolysis of anhydride linkages is inhibited by acids, so bulk erosion of polyanhydrides is autosuppressed by generation of diacid hydrolysis products and erosion process is biased towards outer surface of polymer

4.6 Polyphosphazenes

Polyphosphazene is a bioinert material consist of two different types; a

hydrophobic surface and a hydrophilic surface character. However, polyphosphazene bearing fluoroalkoxy side groups are some of the most hydrophobic synthetic polymers known. These polymers are elastomeric or flexible, easy to prepare and can be used as coating for other materials. Furthermore, hydrophobic polyphosphazenes are potential candidate as erodible biostructural agents for sutures or as matrices for controlled drug delivery systems (Allcock, 1990).

This polymers are most commonly synthesized by a substitution reaction of reactive polydichlorophosphazene with a wide range of reactive nucleophiles such as amines, alkoxides and organometallic molecules. The reaction is carried out in tetrahydrofuran or aromatic hydrocarbon solutions at room temperature. The schematic diagram of polymers containing mixed substituents obtained from sequential or simultaneous reaction with several nucleophiles illustrated in figure 25.

The property of polymers depends upon nature of side groups. The biodegradation of polyphosphazene displays an uniqueness that the inorganic backbone (stem) which in the presence of appropriate side group such as amino acid ester or imidazoyl is capable undergone facile hydrolysis to phosphate and ammonia. The phosphate can be metabolized whereas ammonia is excreted, then polymer is eroded under hydrolytic condition. The first synthetic polyphosphazenes were ethylglycinato derivatives, polyphosphazene containing amino acid ester side group. They are solid materials which hydrolytically degrade to ethanol, glycine, phosphate and ammonia. They have been used for controlled release drug delivery system such as antiinflammatory agents and steroids. The evidence of irritation, tissue inflammation, cell toxicity or giant cell formation does not found when used these polymers (Grolleman, 1986). Imidazoyl – substituted polyphosphazenes are good

biocompatibility but are very easily hydrolysed. The hydrolysis rate can be slowed by incorporation of hydrophobic side groups such as phenoxy or methylphenoxy.

4.7 Polyamides

The amide - based polymers especially natural proteins has been utilize in pharmaceutical application as biodegradable matrices in recent years. Microparticles produced from natural biodegradable polymers such as collagen, gelatin and albumin have been used as drug delivery and from polyamides such as polyglutamic acid, polylysine and their copolymers with various amino acids have been used as drug carrier (Kohn, 1990). Natural polymers remain primarily attractive despite the advent of synthetic biodegradable polymers because they are natural products of living organisms, readily available, relatively inexpensive and capable of a multitude of chemical modifications (Bogdansky, 1990).

Collagen is a major structural protein found in animal tissues in which it is typically present in form of aligned fibers. It is biocompatibility and nontoxicity for most tissue, so it has been used in many biomedical applications as absorbable sutures, sponge wound dressing, and as drug delivery systems especially microspheres. However, some factors such as antigenic responses and tissue irritation due to residual aldehyde crosslinking agent have limited its use as a drug delivery device. Hence, noncollagenous proteins, particularly albumin, continue to be developed as drug delivery vehicles because of nontoxicity, nonimmunogenicity and biodegradability into natural products.

Pseudopolyamino acids, a new approach for biomaterials based on amino acids had been investigated by Kohn and Langer (1985 and 1987). They synthesized poly–

N-acylhydroxyproline esters and tyrosine–derived poly–iminocarbonate as described in figure 26.

Pseudopolyamino acids belonging to polyamide group represent one of biodegradable polymers for medical use as well as controlled delivery of drugs ranging from biodegradable bone nails to implantable adjuvants. They are no gross toxicity, no inflammatory reaction or tissue incompatibility. However, only a few of them have been synthesized and characterized and clinical tests have so far been conducted.

Chitin and chitosan

A natural polymer, chitin, a long straight chain polysaccharide constituted of 2-acetamido-2-deoxy-D-glucose whose units are linked by β- (1,4) glycosidic bonds as depicted in figure 27. is one of the most abundant polysaccharides found in nature second only to cellulose. It is a structural component of exoskeleton ot crustacea, insects and some fungi. The principal derivative of chitin is chitosan, usually obtained by alkaline deacetylation. A schematic diagram of production process of chitin and chitosan is illustrated in figure 28.

Chitosan, (1,4) – 2 – amino – 2 – deoxy -β-D-glucan, is a biopolymer with a repeating unit of disaccharides as depicted in figure 27. It is insoluble in water, organic solvents, alkalies and mineral acids (Muzzarelli, 1973). It is soluble in formic acid, acetic acid and citric acid. Further, inorganic acids can dissolve chitosan at certain pH values after prolonged stirring and warming. Nitric acid can dissolve chitosan but sometimes after dissolution one can observe a jelly white precipitate. Hydrochloric acid also requires heating and stirring for hours. Sulfuric acid does not dissolved chitosan because it forms chitosan sulfate which is a white crystalline solid.

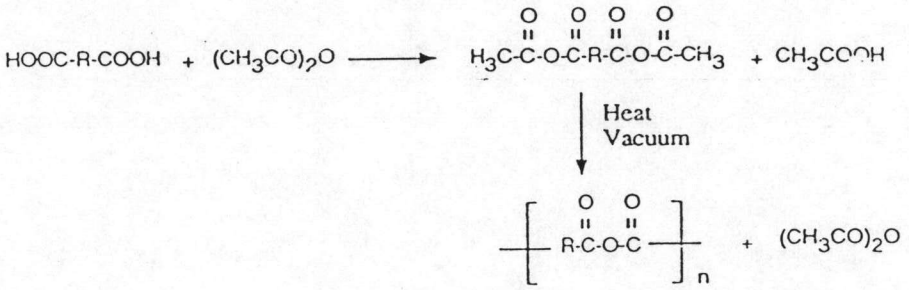


Figure 24 Schematic diagram of polyanhydride synthesis

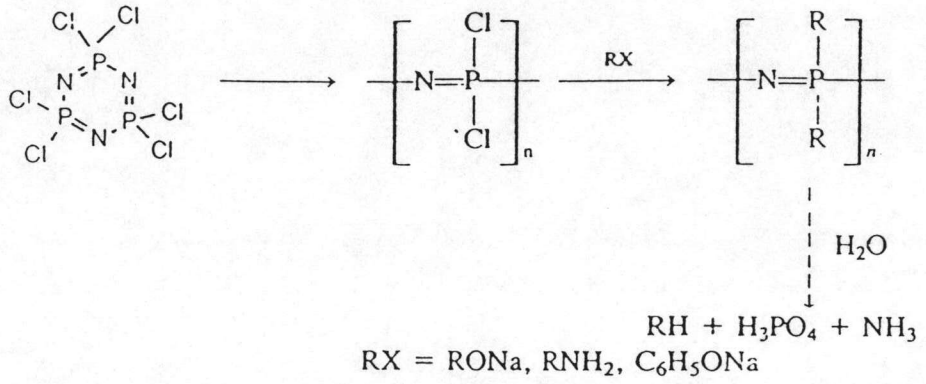


Figure 25 Synthesis and hydrolysis of polyphosphazenes.

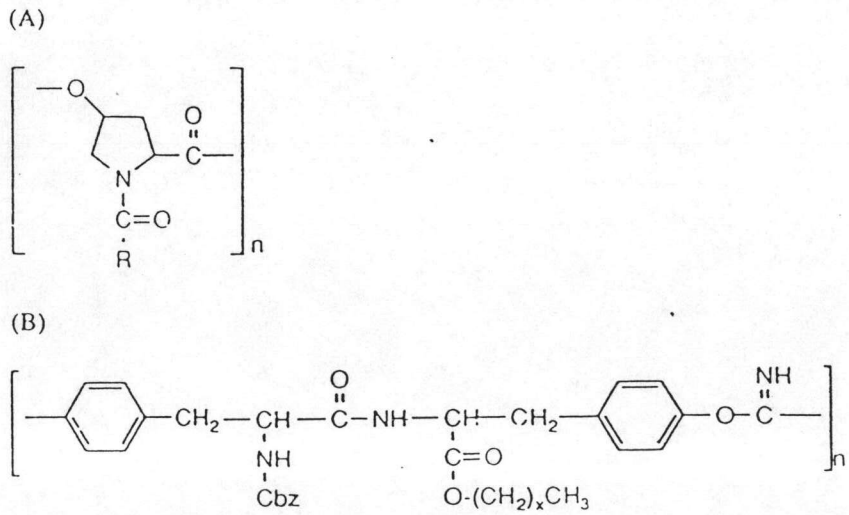
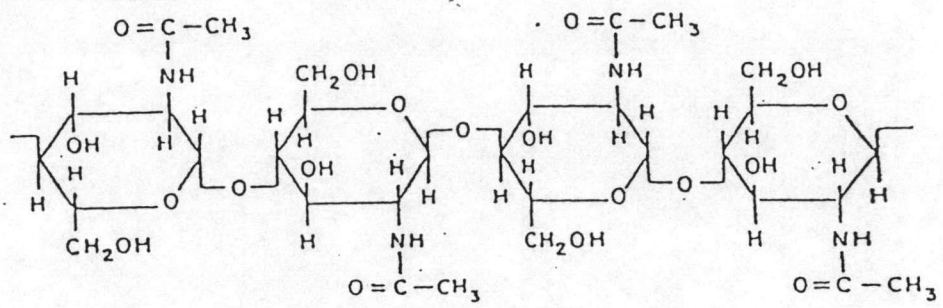
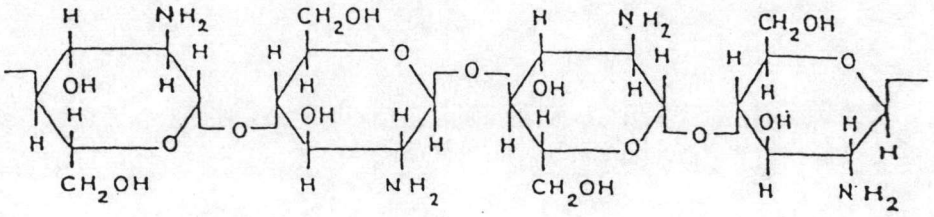


Figure 26 Molecular structure of pseudopolyamino acids. (A) Poly-(*N*-acylhydroxyproline esters); (B) Tyrosine-derived polyiminocarbonate, poly-CTTE, $x = 1$; poly-CTTH, $x = 4$; poly-CTTP, $x = 15$.



chitin



chitosan

Figure 27 Structure of natural polymers : A) chitin , B) chitosan

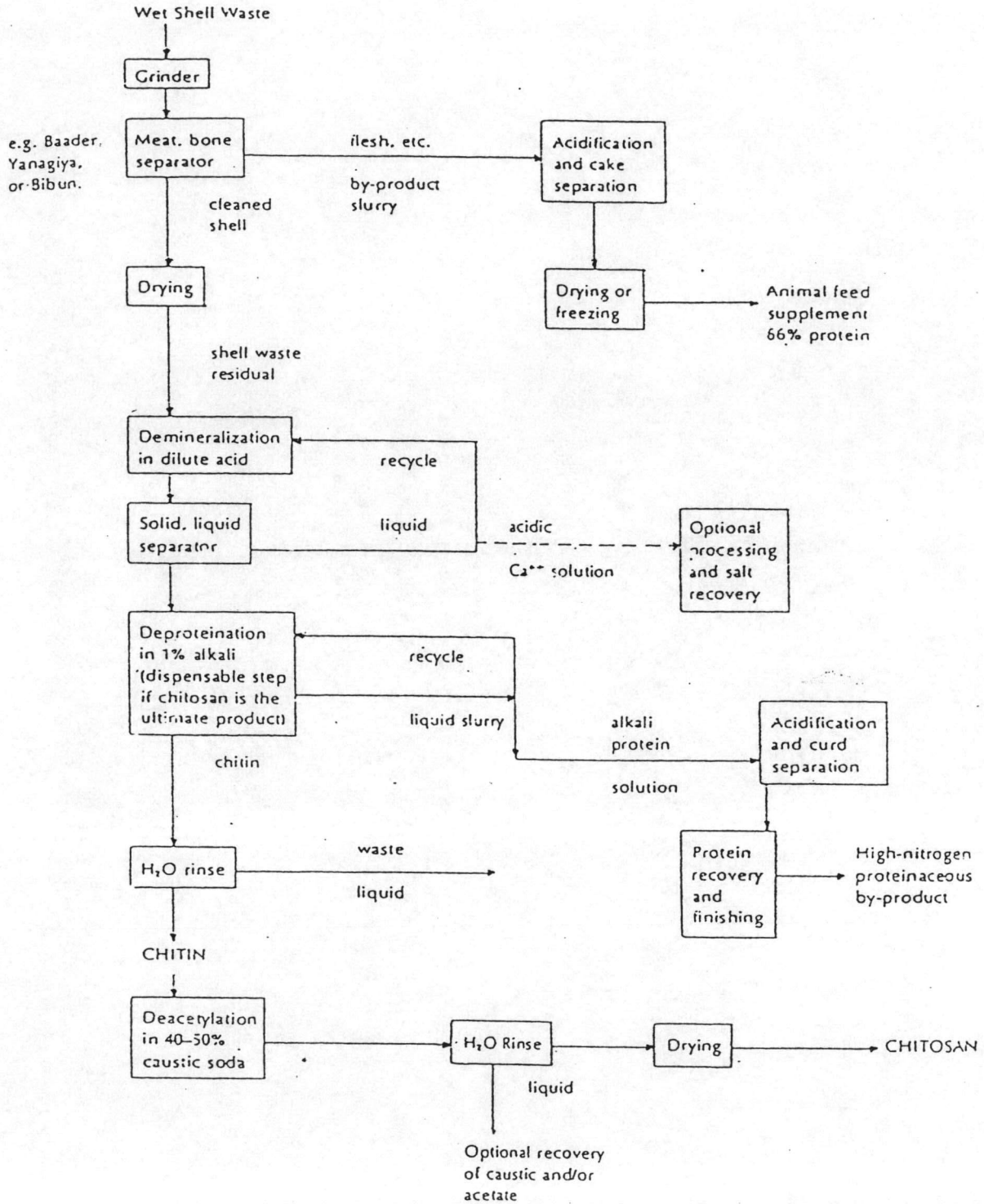


Figure 28 Schematic diagram of production process of chitin and chitosan

Chitosan, a biopolymer with unique properties, can be utilized in a variety of ways. Commercial chitosan is a particularly exciting polysaccharide for several major reasons such as abundant potential resources, unique material characteristics and various functional properties (Rha, 1982). The molecular weight of chitosan depends on processing conditions and more grade within range of 10,000-1,00,000 dalton can be available. The mole fraction of deacetylated units, glucosamine, defined as degree of acetylation, is usually range from 70 to 90 percent. The degree of deacetylation can be varied according to the intended use (Karlsen, 1991).

Chitosan exhibits favorable biological properties including biodegradability, biocompatibility and nontoxicity (Muzzarelli, 1977). So it is beneficial to medical and pharmaceutical applications. It is reportied to have pharmacological properties such as wound-healing properties (Sparkes, 1986), antacid and antiulcer activity (Hillyard, 1964), hypocholesterolemic action (Furda, 1980) and its polycationic character gives its ability to bind strongly to several mammalion cells, resultimg in many potential uses such as hemostatic (Malette, 1984). In addition, its cationic character along with the presence of reactive functional group has been a feaseability in controlled-release technology. The polymer properties especially molecular weight and degree of deacetylation significantly affect pharmaceutical formulation properties.

Chitosan has been greatly developed as a potential vehicle for drug administration for many years. The early use of chitosan is a tableting excipient. It has been mainly used as direct tableting agent (Sawayanagi, 1982 and Knapczyk, 1993), binder (Upadrashta, 1992), and potential disintegrant (Ritthidej, 1994). A furthur advantage is possibility to administer ulcerogenic drugs such as aspirin and other antiinflammatory drugs (Felt, 1998) owing to the gel-forming property of polysaccharide at low pH made it to prevent irritation in stomach. More recently,

chitosan had been managed as bioadhesive system (Lehr, 1992 and Miyazaki, 1995). This system reveals neither irritation nor unpleasant taste or discomfort and moreover it show tight adhesion of adhesive tablet with mucosa in sublingual site and great enhancement of bioavailability of drug after administration. In addition, chitosan has been used as bioerodible matrix, granules and/or beads (Hou, 1985, Kawashima, 1985 and Bodmier, 1989) to provide sustained release of drug owing to its gel-forming ability at low pH. In vitro data, it expressed a near zero-order release from chitosan beads and a first-order release from chitosan granules. The mechanism of drug release is probably diffusion from beads while it is disintegration of matrix in granules. However, chitosan beads or granules can delay, prolong and give higher plasma level of drug after administration. Another possible way to provide sustained drug release is to place implants in body tissues. Chitosan is one of polymer that can be used as implantable dosage form owing to its characteristics including biodegradability, nontoxicity, sterilizability, good mechanical properties, and ease of film formation. In vitro and in vivo studies concluded that in vitro seemed to provide a relatively good model for in vivo behavior, even though degradation in vivo was faster than in vitro. This could suggest that degradation of chitosan involve in enzymatic degradation. Despite its well-known film forming property, the usefulness of chitosan membrane as transdermal device has a few reports. Thacharodi and Panduranga Rao (1993 a,b) assess efficiency of chitosan membrane as rate controlling membrane using hydrophilic and hydrophobic drugs. They concluded that water-soluble drugs could be transported through membranes predominantly via a pore mechanism. Hydrophobic drugs would be affected by both pore and partition mechanism. Moreover, chitosan is enable to interact with liposome. Two excellent effects is obtained, one is a stabilization liposome and the other is a possibility to target liposome vesicle to a specific site due to its mucoadhesion property (Henriksen, 1994). Thus, chitosan-coated liposome is a one interesting approach for administration of poorly absorbed drugs as well as peptides.

Over the last few decades, the systems of delivering active substances to a specific target site have been investigated. The microparticle including microcapsule and microsphere is one approach to be considered as a good delivery system. Because microparticles not only are intended to provide sustained drug release but are also localized active drug to target site. Many polymers have been used to produce microparticle including synthetic polymers as described above and natural polymers such as chitosan. A great deal of chitosan microparticles described in many literatures have been prepared using a suspension or emulsion cross-linking process (Thanoo, 1992) and a precipitation process by using sodium sulfate as precipitant (Berthold, 1996). In addition, chitosan microparticles can be prepared by using chitosan-calcium alginate microencapsulation process (Polk, 1994). This approach is more generally used than other methods such as precipitation, interfacial polymerization or spray drying due to polyelectrolyte chitosan has been interacted with counterion, sodium alginate, in presence of calcium chloride and no effect of organic solvent. Moreover, chitosan-alginate membrane can control release rate of active material and protect substance from denaturation and degradation when oral administration. However, preparation parameters such as nature and ratio of polymeric materials, size, shape and density of microparticles, nature and amount of cross-linking agent and physicochemical interaction between drug and polymer are largely influenced morphological and biopharmaceutical characterstic of microparticles. Furthermore, molecular weight and degree of deacetylation of chitosan are significantly affected particle size, drug content and entrapment efficiency of microparticles.

The drug release from chitosan microparticle is characterized by an initial burst effect which is probably due to release of adsorbed drug on surface of microparticles (Miyazaki, 1986). This rapid initial phase can be reduced by addition of some

substances such as chitin, alginic acid, stearic acid and sodium salt of caprylic acid. Several factors including type and concentration of chitosan, drug loading, viscosity of lipophilic phase, cross-linking density and microparticle size has affect drug release from chitosan microparticles.

Many literatures support that chitosan microparticle as drug carrier to control delivery over very long periods and avoiding repeated administration of certain drugs. A great number of active materials such as griseofulvin (Thanoo, 1992), theophylline (Lin, 1992), ketoprofen (Genta, 1998), prednisolone sodium phosphate (Berthold, 1996) and cancer chemotherapeutic agents (Hassan, 1992 and Wang, 1996) had been reported to encapsulate in chitosan microparticles.

In addition, chitosan also has utility in delivery of peptides and proteins due to high biodegradability, nontoxicity, neither irritant nor allergen in humans. Moreover, chitosan has biological properties including immune system stimulant (Sandford, 1989). Many researchers have been investigated the immune stimulating activity chitosan preparations. Chitosan suspensions or microparticles had been reported to have immune stimulating activity such as increasing accumulation and activation of macrophage and polymorphonuclear cells, suppressing tumor growth, promoting resistance to infections by microorganisms, inducing cytokines, augmenting antibody responses and enhancing delayed type hypersensitivity and cytotoxic T lymphocyte responses (Seferian, 2001).