CARBOHYDRATE NANOPARTICLES: A NOVEL DRUG DELIVERY PLATFORM

FOR THE SYSTEMIC ROUTE

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VITA

Arindam Basu Sarkar was born on the 5th of April, 1968 in Calcutta, West Bengal, India to Mr. Suhaskanti Basu Sarkar and Mrs. Mandira Basu Sarkar. After completing his school education from Jadavpur High School, Calcutta in 1986, he commenced university education in Department of Pharmacy, Faculty of Engineering, Jadavpur University. He graduated with Bachelor of Pharmacy in 1991 and started working in pharmaceutical industry. Following a two year stint (September 1995 to October 1997) in Himalayan Pharmacy Institute as a lecturer, he commenced his Masters in Pharmaceutical Engineering in Jadavpur University. Upon obtaining his Masters in 1999, he started pursuing graduate education in Pharmaceutics at Harrison School of Pharmacy, Auburn University, Alabama. During his studentship in Auburn University, he served as Graduate Teaching Assistant as well as Graduate Research Assistant in the pharmacy school. In May, 2005 he received 'Don Tillery Scholarship' from Harrison school of Pharmacy. In April, 2006 he received 'Outstanding Doctoral Student' award from the Graduate School, Auburn University for the academic year 2005-2006. He is also recipient of 'Postgraduate Best Paper Award' in the basic sciences section of American Pharmaceutical Association Annual Meeting and Exposition, 2003 held at New Orleans, Luisiana.

DISSERTATION ABSTRACT

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With the phenomenal developments in the fields of biotechnology and molecular biology, the focus in the medical fraternity is shifting towards cellular level intervention. The use of polypeptides, proteins and possibly genes through targeted delivery platforms is viable today. Available particulate delivery platforms are inadequate targeting platforms because of the poor physical and chemical properties of the polymers used. In course of current research a novel nanoparticulate delivery platform has been developed with hydrophilic carbohydrate polymers, using a water-in-oil emulsion and interfacial crosslinking. Synthesis of nanoparticles has been characterized and key parameters affecting the reaction identified. The reaction is dependent on the pH, species of the polymer, the polymer load of the dispersed aqueous phase and species of the crosslinker.

Smaller starch polymers were found to be most suitable for this purpose because their relatively higher aqueous solubility and superior rheological properties. Trimesoyl chloride was found to be the best crosslinker because of better reactivity and the higher hydrophilicity imparted to the surface. Sorbitan mono palmitate (Span 40®) was found to be the best surfactant. The nanoparticles have been chemically characterized by determining the degree of substitution (DS). Analytical methodology was developed for quantifying the DS. The reaction occurs in a wide pH range of 3-11 and was base catalyzed at higher and acid catalyzed at lower pHs. The nanoparticles prepared using maltodextrin from amylopectin and trimesoyl chloride had hydrophilic surfaces. The particles suspended in aqueous media as monodispersed mononucleated particles.

The crosslinked membrane of the nanoparticles was largely inadequate in sustaining the release of small molecules like tartrazine. For moderate sized proteins like lysozyme though, the membrane was adequate in sustaining the release for weeks. Diffusion based modeling of release data showed two independent modes of release, one fast phase which is complete within 3 hr and one slow phase lasting for weeks. Estimated permeability coefficients show that there is a negative correlation between the degree of substitution and the permeability coefficient of the slower mode of release. Further work is required to establish the behavior of these nanoparticles *in vivo*. Overall, the developed platform appears suitable for macromolecular delivery in the systemic circulation. Future studies will investigate potential cellular level intervention.

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I. INTRODUCTION

The last two decades have seen a phenomenal growth in the fields of molecular biology and bio-technology. Major advancement, especially in the areas of recombinant DNA technology and hybridoma technology has resulted in a deluge of macromolecules showing therapeutic potentials which are gradually being introduced to clinical studies. Unlike in the past, when polypeptides and proteins from human and animal sources had to be carefully isolated and purified at a laboratory scale, it is now possible to produce commercial quantities of polypeptides and proteins like interleukins, immunoglobulins, growth hormone and insulin, to name just a few, in a high degree of purity, using transcriptional and translational methods in bacteria. Additionally, developments in functional genomics and proteomics are likely to expand the therapeutic repertoire even further, once the gene-protein and protein-protein relationships are firmly established for 5,000-10,000 drug targets expected from the human genome map (Drews, 2000). Already, by 2002, 130 biotechnology derived protein drugs and vaccines were approved by FDA in the US (Biotechnology Industry Organization Site, 2002). Seventy percent of these were approved in the last 6 years. As indicated by the latest available information, some 371 biopharmaceuticals were in clinical trials in the USA (New biotechnology medicines in development report, 2002). More than 300 of them were protein based, with recombinant vaccines and monoclonal/engineered antibodies representing the two

most significant product categories. Over the coming decade, therefore, approximately a dozen new therapeutic proteins should, on average, gain regulatory approval each year. In the similar time frame of 1995-2002, 88 recombinant proteins/monoclonal antibody-based products had gained marketing approval in the European Union (EU). This represents 36% of all new approvals in the EU in that period (Walsh, 2003).

Despite the initial enthusiasm, it remains a fact that no gene therapy based product has thus far been approved in either the USA or EU. Clinical trials have also reported a disappointing lack of efficacy and in some cases have raised safety concerns (Stein, A., 1998; Pfeifer, A., 2001; Phillips, A., 2001; Lebedeva, I., 2001). The failure of nucleic acid based products to meet original expectations in no way reflects a flaw in the concepts of gene therapy or antisense technology. These technologies retain the potential to revolutionize medical practice. Their full potential will however, accrue only when several associated technical difficulties pertaining to stability and regulation of expression and gene/nucleotide delivery are overcome (Walsh, G., 2003).

This brief overview of the drug discovery area shows a significant, steady but gradual change of trend. Increasingly, more and more of the new drug molecules approved world wide are and will be macromolecular in origin. These so called biopharmaceuticals including polypeptides, proteins, macromolecule-protein conjugates and nucleotides present a new and emerging challenge to the science and technology of formulation and/or drug delivery.

Biopharmaceuticals, especially proteins are fundamentally different from low molecular weight drugs. Unlike low molecular weight drugs, the chemical

structure of the proteins, as described by their amino acid sequence (primary structure), does not adequately define them. They have specific native three dimensional structures based on secondary structures (like alpha-helices, beta sheets and random coils, etc.), tertiary structures (folding of secondary structures into complicated three dimensional arrays) and in some cases quaternary structures (where different monomers interact). The secondary, tertiary and quaternary structures of proteins often change with the change in environmental conditions like polarity and ionic strength of the media, pH, temperature and presence of foreign molecules, often leading to irreversible loss of native structure (called denaturation) and thus the activity of the protein. Accurate and absolute quantification of these natural and denatured configurations of therapeutic proteins is difficult and often impossible with currently available analytical tools (Crommelin, D. J. A., 2003).

These conformational complexities aside, proteinacious drugs are also complicated in terms of their stability and effectiveness. Peptide bonds are hydrolyzed in extreme pH ranges, like the acidic pH of media present in sections (stomach) of the gastro-intestinal tract (GIT). Proteolytic enzymes, present in the GIT, can also break down proteins into small polypeptides and constituent amino acids. Protein drugs are fast metabolized by the liver and eliminated from the body. The biological half-life of protein drugs is usually very short, ranging from a couple of minutes to a few hours, while their medical applications frequently require prolonged residence time in the body. There are inter-species variations in chemical structure of biologically active proteins. The structural difference is often enough to cause an immune response. Purity of proteins is also a great

concern. A small amount of bio-molecular impurity, usually coming from expression systems and growth conditions, can impart immunogenicity.

All of these structural and conformational complexities of the therapeutic macromolecules like polypeptides and proteins, set them apart as a completely different category of drugs. They have to be formulated, handled and delivered differently with the materials, methods and route(s) of administration carefully chosen to adequately address these complexities. The classical approach to drug therapy, through oral administration, is thus not applicable for these macromolecules.

Peroral delivery of proteins is hampered by several factors. The main impediment is that they are rapidly hydrolyzed by proteolytic enzymes and the acidic pH in the gut. Even if the proteolysis is controlled and overcome to a manageable extent, the trans-epithelial absorption of molecules beyond the molecular weight of one thousand Daltons is pathetically inadequate. There are emerging approaches directed towards oral delivery of macromolecules. These include stabilization and protection of the macromolecules in the harsh environment of the GIT (Sood, A., 2001) and improving the membrane permeability, either by the use of permeability enhancers (Sharma, P., 1999) or by exploiting the natural carrier system in the GIT (Orelana, I. G., 1998). Despite these developments, a commercial and successful peroral delivery of proteins is still a distant goal.

The significant obstacles associated with the peroral route have forced the focus to other possible routes of drug delivery like transdermal. While the transdermal route has the inherent advantage of simplicity and ease of application, it also presents enormous challenges and limitations. Penetration of drugs through the stratum cornium is

inadequate, even for most small molecules. There seems to be a critical need of optimum polarity and molecular weight of the drug molecules for adequate permeation through the transdermal route. This, in turn, limits the applicability of the route for drug delivery. Since the introduction of the scopolamine patch in 1983, only eight drugs have been commercialized in the next seventeen years, proving that drug delivery through the skin has limited utility (Naik, A., 2000). Chemical and physical enhancement techniques like iontophoresis, electroporation, sonophoresis and high speed powder delivery are under active investigation to improve the scope of drugs (in terms of size, lipophilicity and charge) that can be delivered through the skin (Pilli, O., 2001). Transdermal delivery of proteins and polypeptides with all these chemical and physical enhancement tools are still experimental technologies with many unresolved issues like skin safety considerations (Panchagnula, R., 2000). They are far from reaching a stage of critical maturity and are unlikely to impact macromolecule delivery significantly in the foreseeable future.

Another promising route for macromolecule delivery is nasal mucosa. Of all the mucosal tissues, nasal mucosa is considered to be most permeable and offers potential for peptide delivery (Patridos, C. D., 2000; Brayden, D. J., 2000), particularly for vaccines. Already several peptide formulations are on the market and further development will depend on minimizing the immunogenicity of the bioactive agents and the long-term effect of the permeation enhancers on the nasal mucociliary activity.

The lung also offers a potential opportunity for systemic delivery. It has enormous absorptive surface area comparable to that of a tennis court and is rich in blood supply (Gonda, I., 2000). This route is targeted through reservoir-type micro/nano particulate drug delivery devices. However, the major limitations are high dose requirements, low

efficiency of deposition on alveolar surfaces, and the reproducibility of metered doses. Although it is possible to design particles close to the desired characteristics for aerosolization using supercritical fluid technology (York, P., 1999), further progress awaits improved aerosol design. The new generation of breath activated dry powder inhalers are an improvement in that direction (Ashrst, I., 2000).

Progress is being made in the delivery of macromolecules including the proteins and polypeptides. Nevertheless, in spite of a few marginal exceptions, an overwhelming majority of the macromolecules are being administered today by intra-venous bolus injections. Due to the short plasma half-life and the usual need for chronic treatment with these drugs, repeated intra-venous bolus injections are used. This mode of treatment suffers from a number of disadvantages including inconvenience, lack of patient compliance, need of hospitalization or professional care and most importantly high cost of treatment. The search for alternative routes and delivery systems for proteins and polypeptides are all aimed at eliminating or minimizing some or all of these disadvantages. From this point of view, obviously, absorbable oral formulations are the ideal answer. But unfortunately, that is not feasible with the current level of technology. Of the other routes, the pulmonary and injectable routes currently show most promise. In both these routes drug release is controlled and sustained by incorporating the drug(s) within micro/nano meter sized, reservoir-type, polymeric particles. This current research is aimed at developing the processing technologies for making such micro/nano particles, overcoming some of the limitations associated with the current technologies for making such particles and establishing their effectiveness with respect to macromolecules and investigating their effectiveness with and possible use for small drug molecules. In the

next section, the status of prevalent current technologies, their merits and limitations, the choice of appropriate polymers for such technologies and devices will be explored with respect to their applicability and usefulness in the current context.

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II. LITERATURE REVIEW

1. INTRODUCTION

Over the last three decades, particulate drug delivery systems have been the central focus of drug delivery research. An enormous amount of research effort from both academia and industry has been devoted towards the development of these particulate systems to deliver drugs safely, effectively and in a manner that improves the therapeutic efficacy of the drug as well as makes the intervention process more patient-friendly. These delivery systems essentially consist of spherical, biodegradable polymeric devices containing the active drug and with size ranging from nanometer to a few hundred micrometers. As there has been some confusion regarding the terminology, for the purpose of uniformity, spherical particles in the sub-micron range are termed nanospheres and those above one micron are termed microspheres in this text.

Particulate drug delivery systems have been prepared from a host of different bio-degradable and non-degradable polymers, both hydrophilic and hydrophobic. The term biodegradable polymer is defined as a polymer which degrades by either hydrolysis or solubilization in the physiological conditions *in vivo*. These polymeric systems include both non water soluble polymers that are degraded

through surface or bulk-erosion, and water soluble gels that are dissolved and cleared slowly from the body without undergoing a decrease in molecular weight.

Sustained release delivery systems have been successfully prepared in the past using non-degradable polymers such as Implanton® from Organon (Tice, T., 2004). Implanton is a single rod shaped formulation made with ethylene vinyl acetate, a non-degradable polymer. The long-acting contraceptive formulation releases the steroid molecule etonogestrel for three years. But despite the market success of Implanton, non biodegradable polymers are not generally used for the preparation of particulate sustained release delivery platforms mainly for two reasons; (1) the need for implantation and surgical removal of the implants and (2) the concern about long-term histo-toxicological effects of the non degradable platforms. Consequently, almost all current developments in this area are carried out with biodegradable polymeric systems, though there is still some interest in non biodegradable polymers in the area of sustained local delivery of drugs.

Significant progress has been made in the sustained release systemic delivery of drugs using biodegradable polymers, both through particulates and implants. All such commercial successes involve the use of co-polymers of lactic acid and glycolic acid (PLGA) in various combinations of different proportions of the monomers and also the molecular weight of the overall polymer. Currently there are 15 different PLGA depot formulations in the US market (Tice, T., 2004), involving 13 drug companies and 10 different drug molecules. These products deliver small molecular weight antibiotics (doxycycline, minocycline), antipsychotic (resperidone), small polypeptides (LHRH analogs triptorelin, luprorelin) and one large molecular weight protein, growth hormone,

for a time frame of one week to four months. Many PLGA depot formulations are being developed today. Products in preclinical and clinical stages include formulations of naltrexone and buprenorphene for alcoholism/drug abuse/terminal pain treatment, GLP-1 peptides for diabetes, r-hFSH for fertility, dopamine for nerve growth, dexamethasone for ocular application, melanotan and plasmid DNA for cancer prevention, a variety of vaccines and the generics of luprorelin and octreotide.

As evident here, micro/nano particulate sustained release delivery systems have come a long way from being just another new idea in drug delivery research. The idea has been proved to be effective and achievable beyond doubt by the commercialization of the products enumerated above. It is now well accepted that traditional small molecular drugs can be effectively incorporated in the particulate devices and released in the body in a sustained manner and the release rate can be potentially tailored according to the need. The application of this technique to small molecules will be limited only by pharmacological profiles of the small molecules and the clinical needs. Currently in most clinical situations, long term treatment with traditional small molecules is simply achieved through multiple oral dosing (though the issue of lack of patient compliance remains unresolved). Development of sustained release formulations of these molecules are theoretically possible but in most cases not driven by the market because of cost considerations. But there are some special clinical situations like long term de-addiction therapy with opiates like buprenorphene, where a sustained release injectable delivery system is the only option because of the concerns of drug abuse (Mondal, T., K., 1999). Also, injectable particulate delivery systems for sustained release are the only way to

achieve long term constant plasma concentration of some small molecular weight drugs that show inconsistent and low bioavailability and short half-life.

Another important application of particulate delivery systems is in the delivery of proteins and polypeptides. Quite a few small polypeptides are currently in the market. These polypeptides, mainly leutenizing hormone releasing hormone and follicle stimulating hormone analogs, are small in size and thus lack the secondary, tertiary and quaternary structures. Thus they are relatively easier to formulate since the problem of denaturation and consequent loss of activity does not have to be dealt with for these molecules. The enormous challenge lies in the delivery of big protein molecules while preserving their native structures and activity. A modest beginning has been made by the commercialization of a single growth hormone formulation. Still generalized use of sustained release injectable delivery systems for the delivery of bigger proteins through the use of particulates remains elusive. A more thorough understanding of the micro-environmental requirements and restrictions imposed on the preparation process and delivery platform by the complicated structures of the big proteins are needed, potentially in addition to newer approaches to address these issues.

Particulate delivery systems can also be potentially used in other emerging areas like gene therapy. In this field, disease conditions are approached from the perspective of genetic correction of the physiological system through the introduction of a DNA fragment into the cells (usually in the form of plasmids with appropriate helper domains). But unfortunately there are no effective ways of introducing the plasmids into cells *in vivo* in the clinical environment. The currently used gene transfection vectors like adeno virus, adeno-associated virus (Monahan, P. E., 2000), cationic lipids or lipoplexes (Ferrai,

S., 2002) and low molecular weight citosan (Gao, S., 2003) are not effective in gene-transfection. In addition, there are other problems peculiar to each of the platforms. Particulate delivery systems have been shown to be taken up by different mammalian cell lines presumably by endocytosis and fluid phase pinocytosis (Desai, M., 1997; Wilhelm, C., 2003). It is also established that the uptake is size dependent, the smaller the size the higher is the efficiency of uptake (Desai, M., 1997; Prava, S., 2002). Other factors influencing the uptake include the surface polarity, surface charge and presence of some receptor-specific molecules on the surface of the particulates (Wilhelm, C., 2003). Experiments have also proved that uptake of bio-degradable PLGA nanoparticlates into the cell does not affect the cell viability (Davda, J., 2002). Thus particulate delivery systems, especially the nanoparticles, have potential as DNA delivery vectors. This application is currently being pursued seriously.

Particulate delivery systems seem to be fast developing into a basic platform which can be used to serve different purposes with appropriate modifications. Currently long circulating reservoir type applications are not technologically feasible. The successful systems involving PLGA as polymers are limited by the surface properties that activate the natural immune system of the body and quickly eliminate the particulate drug delivery system (DDS) (Stolnik, S., 1995). This characteristic of the PLGA particulates limit their applications to intra-muscular or subcutaneous depot forming formulations. However, this property can be exploited in specific cases where the targeted site is the immune system itself, for example in acquired-immuo-deficiency syndrome (AIDS) treatment. Long circulating reservoir type application is possible with this platform if the surface can be adequately modified to escape the immune system. Once that is

technologically possible, long circulating particulate depots can be targeted passively by the use of magnetic field or actively by antibody targeting to the site of action. This platform can also be used in imaging applications by incorporating appropriate molecule(s). It has the promise to affect a paradigm shift in a broad spectrum of medical technologies as it matures over time.

2. GENERAL CONSIDERATIONS

Since the particulate DDS has to function in a dynamic physiological environment, enormous complexity is involved in determining the overall applicability, efficiency and success of the systems. The multitude of factors affecting the particulate DDS can be grossly classified into two groups; the physical factors and the physiological factors.

2A. PHYSICAL FACTORS

The physical factors affecting the particulate DDS include the polymer related factors, the active ingredient related factors and the process related factors. It is important to note that while some of these factors are independent, most have a very complex interdependent relationship with one another.

2A (*i*) THE POLYMER RELATED FACTORS

The polymer is the most abundant component of the particulate DDS. The properties of the polymer determine the behavior of the DDS in physiological conditions to a large extent. The chemical structure, stereochemistry, crystallinity and molecular weight are important factors that determine the polymer properties in this context.

Generally speaking, the more non-polar the constituent monomer, the slower is the degradation of the resulting polymer in the physiologic conditions. A common example is the erosion controlled particulate DDS composed of poly(lactic/glycolic) acid. Lactic acid is more hydrophobic than glycolic acid. The micro/nano spheres fabricated with poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) of comparable molecular weight show different rate of polymer degradation accompanied by different drug release kinetics. On exposure to water, the bulk erosion polymers like PLGA and PLA undergo random chain scission by simple hydrolysis of the ester bonds following hydration of the polymer. Thus PLA being more hydrophobic than PLGA, the extent of hydration and consequently the extent of degradation of the former in physiological conditions is less. This results in a slower release of drugs from PLA spheres than PLGA spheres (with polymers of same molecular weight and specific area). The effect of molecular weight and copolymer ratio on the extent and rate of drug/macromolecule release has been reviewed (Ogawa, Y., 1988). Since, the drug/macro-molecule has to be released into the body-fluid, hydration of the polymeric surface of the devices is important not only for degradation of the polymeric spheres but also the actual release kinetics. The more the hydrophobicity, the less the hydration and also the less is the release rate. The polymer polarity also determines the solubility profile of the polymers. All the commonly used polymers for sustained release DDS are moderately to highly non-polar and thus are soluble in solvents of moderate to high degrees of non-polarity. These solvents are generally not compatible with biological macromolecules. Thus the polarity or hydrophobicity of the polymer is a critical constraint in selection of a solvent

system which is benign enough with regards to its chemical activity on the bioactive molecule to be used to form the DDS.

In addition to composition and molecular weight, other factors such as crystallinity and stereo-regularity of the polymer, affect its polarity and solubility. While low molecular weight polymers tend to be more hydrophilic and degrade faster than high molecular weight polymers (Kwong, A. K., 1986) the stereo-regularity of the monomer units affects the tightness of packing of polymer chains. The more the stereo-regularity, the tighter is the packing of polymer chains which become more crystalline and less amorphous. The crystallinity of the polymer disfavors its degradation and slows down drug release kinetics (Migliaresi, C., 1994). Thus poly-L-lactide is crystalline while poly-D, L-lactide is amorphous and the degradation time of the former polymer is longer than the second one (Sinha, V. R. 2003).

Another important factor in the selection of the polymer is its bio-degradability. The rate of degradation may also be an important factor in the selection process as it might be used to control the drug release rate in erosion controlled systems. There are several kinds of labile bonds that are used to form biodegradable polymers. These bonds may be categorized by their relative rates of hydrolysis under physiological conditions based on the known hydrolysis rates of low molecular weight analogs (Baker, R., 1987) as shown below.

Polycarbonate > *Polyesters* > *Polyurethanes* > *Polyorthoesters* > *Polyamides*

Since polymer morphology and the presence of substituent groups can influence the rates of hydrolysis significantly, the above comparison is only an approximate guide. Biodegradable polymers containing almost all of the above mentioned bonds have been used to produce particulate DDS in an effort to achieve a required level of balance between release rate, biodegradability, biocompatibility, toxicity and encapsulation efficiency. Examples include bulk erosion polymers like PLA, PLGA blends, block co-polymers of PLGA and PEG (polyethylene glycol), poly(cyanoacrylates), surface erosion polymers like poly(anhydrides) and poly(orthoesters), hydrogel systems like polyols (pluronics), poly(vinyl alcohol), poly(vinyl pyrrolidone) (PVP), hyaluronic acid derivatives, alginate, collagen, albumin, starches and dextrans (Gombotz, W. R., 1995).

Another important issue to be considered is the biocompatibility of the polymers. Polymers used for particulate DDS need to exhibit biocompatible characteristics in terms of both the polymer's effect on the organism receiving the DDS and the polymer's effect on the macromolecule or protein incorporated. Several aspects of polymeric DDS ultimately contribute to its overall biocompatibility, or lack thereof. The polymer itself may potentially be antigenic (De Lustro, F., 1990; 1986), carcinogenic (Nakamura, T., 1994; Weiss, W. M., 1991), or toxic (Yoshida, S. H., 1993; Busch, H., 1994). The shape of an implanted material has been implicated in its biocompatibility as well. Smooth surfaces are less irritating and more biocompatible than rough surfaces (Matlaga, B. F., 1976). One key factor influencing the biocompatibility of the polymer is the presence of low molecular weight extractables. These extractables generally come from the un-reacted monomers and/or polymerization initiators. The monomers as well as the polymerization initiators can be toxic at times. For example, acrylic acid and methacrylic

acid are toxic, while their polymers are relative non-toxic (King, D. J., 1989). These extractables are generally removed by organic solvents prior to manufacture or preparation of DDS. However, the organic solvents used to remove the extractables are themselves not biocompatible and can be a cause of concern by themselves. In some cases, soluble polymers or their breakdown products are sequestered within organs, resulting in long term adverse effects. The accumulation of high molecular weight PVP in liver following prolonged exposure is one such example (Roske-Nielsoen, E., 1976; Meijer, A.E.F.H., 1963). All these polymer related issues have to be considered for selection of the appropriate one for a specific DDS application.

2A (*ii*). THE MACROMOLECULAR DRUG RELATED FACTORS

Incorporating low molecular weight drugs in polymeric particulate DDS and attaining sustained release has been achieved. Macromolecular drugs, especially proteins, however, behave quite differently in these systems. Generally they give rise to a multitude of additional complications resulting in poor encapsulation efficiency, denaturation and concomitant loss of potency, and incomplete release among others. There are a number of different properties and factors peculiar to the macromolecules that lead to these drawbacks.

The first factor to be considered is the protein molecular weight. As the molecular weight of the macromolecule increases, the hydrodynamic radius increases. The diffusion coefficient of the solute is inversely related to the hydrodynamic radius of the solute molecule. As the molecular weight and consequently the hydrodynamic radius of the solute molecule increases, the diffusion coefficient of the solute decreases

logarithmically (Colton, C. K., 1971). So, as the molecular weight of the protein drugs increase, their release rate decreases dramatically. The low diffusivity of high molecular weight proteins, while not unduly restrictive, has to be factored into the design of the DDS.

The isoelectric point of the protein is an important parameter determining the ionization state of the protein at a particular pH. The ionization state of the protein governs ionic interactions of the individual protein molecule with other protein molecules as well as with the polymer in some cases, where the polymer is also capable of being ionized. Thus formation of dimers and oligomers of the proteins and also adsorption of the protein to the polymer surface partially depends on its isoelectric point and the pH of the microenvironment. Additionally, at the isoelectric point, the protein has its lowest aqueous solubility. This also restricts the pH range of operability.

Sulphur containing amino acids like cystine is another source of concern for stability. Cystine is known to participate in covalent interactions like disulphide bonding both intra and inter molecularly. This interaction is often responsible for the formation of oligomers and concomitant denaturization of the molecule and loss of potency. Aggregation of lyophilized formulations of albumin, β-lactoglobulin and glucose oxidase is attributed to disulphide interchange (Johnson, O. L., 1999). Other chemical degradations like oxidation (Pearlman, R., 1993) and deamination are also possible. Deamination is known to cause reduction of catalytic activity of lysozyme (Manning, M. C., 1993) and ribonuclease at high temperatures (Zale, S. E., 1986).

Protein stability in particulate DDS is partially determined by their primary sequence in various ways. The primary amino acid sequence determines the hydrophobic domains

of the protein. The hydrophobic domains are often implicated in protein adsorption on the polymer surface. Human serum albumin is known to undergo multilayer adsorption to PLA nanospheres. At least a fraction of the human serum albumin, adsorbed on the PLA surface, is known to be adsorbed irreversibly (Verrecchia, T., 1993). Protein adsorption to polymeric materials has been widely studied in the context of polymeric implants (Horbett, T.A., 1987). The deleterious effects of protein adsorption to polymeric surface are significant in the context of polymeric particulate DDS containing proteins as well. Attempts have been made to address the problem of protein adsorption on polymeric surfaces by addition of stabilizing excipients (Liu, W. R., 1991; Liu, W. R., 1995; Constantino, H. R., 1994). The primary amino acid sequence of the protein is also important in that it partially determines the possibility of chemical modification/reaction when in association with polymeric materials.

The presence or absence of carbohydrate in association with proteins is known to alter the interactions of proteins with polymer surfaces. Additionally, the presence of a carbohydrate also affects the hydrodynamic volume (Gombotz, R., 1995).

There are additional factors that are encountered regularly in development of particulate DDS. When particulate DDS containing proteins are administered, the incorporated proteins may become hydrated at relatively high concentrations for prolonged periods of time. Proteins in this kind of environment are susceptible to denaturation and aggregation (Liu, W. R., 1991). Additionally, polymer degradation is induced. The degradation products are highly concentrated in the micro-environment. These degradation products can be a source of protein instability including degradation and/or chemical modification of the proteins, especially in situations where the polymer

degradation products change the pH of the microenvironment significantly. In cases where the protein itself has significant moderately hydrophobic domains, in addition to adsorption to polymeric surface, the problem of inadequate protein hydration is often encountered. This leads to incomplete protein release as well as protein aggregation and denaturation. This problem has been addressed by addition of solubilizing or hydration additives (Ogawa, Y., 1988; Okada, H., 1991).

In a different approach, modification of the protein molecule itself and the polymer by introducing water soluble substituent has been attempted to prevent protein aggregation (Delgado, C., 1992) and/or adsorption (Cohn, D, 1988; Sawhany, A. S., 1994). Protein modification with PEG has been accomplished utilizing at least two proteins in PLGA delivery systems; interleukin-2 (IL-2) (Hora, M.S., 1990) and granulocyte colony stimulating factor (C-GSF) (Camble, R., 1994). In both cases, the unmodified protein exhibited poor release profile and much of the protein remained trapped within the polymer after several weeks of incubation in solution. The poor release was attributed to difficulty in resolubilization of the protein. The PEG modified proteins, however, were released much more readily from the systems, probably due to increased solubility, decreased aggregation, and decreased protein adsorption on the polymeric surfaces. All the above mentioned factors related to the macromolecular drug contribute to the overall performance of the particulate DDS and thus need to be taken into account in their design.

2A (*iii*). THE PROCESS RELATED FACTORS

The assortment of processes and techniques used to produce micro/nano sized particles for drug delivery purposes are generally referred to as microencapsulation. The microencapsulation process is generally dominated by the polymer used for the particle formation. Most polymers used and/or investigated for this purpose are hydrophobic. Consequently, a non-polar solvent becomes a requirement for the purpose. Almost all the microencapsulation processes currently in use are variations or modifications of three basic techniques: solvent extraction/evaporation, phase separation (coacervation) and spray drying (Aftabrouchad, C., 1992).

Spray drying is the simplest technique among the three mentioned and has the highest throughput. But this process uses elevated temperature and thus is not suitable for temperature sensitive molecules. Additionally, control of particle size is difficult and yield for small batches is moderate (Johansen, P., 2000). Coacervation, though elegant as a technique, is frequently impaired by residual solvents and coacervating agents remaining in the microsphere (Thomasin, C., 1996). Also, this technique is not well suited for producing microspheres in the low micrometer size range. To minimize the problem of residual solvent and/or coacervating agent, an alternative approach involving supercritical fluid has been tried. This approach has resulted in new processes namely "precipitation with compressed antisolvent" (PCA) (Falk, R. F., 1998), "gas or supercritical fluid antisolvent" ((GAS or SAS) and "aerosol solvent extraction system" (ASES) (Jung, J., 2001). These techniques are in the development stage and are currently limited to very small laboratory batch sizes. Kilo scale or industrial scale production using these techniques have not been reported.

With the obvious limitations of the techniques discussed above, solvent evaporation/extraction seems to be the most appropriate technique to be used for the purpose of preparing/fabricating micro/nano particles to be used as drug delivery platforms. Solvent extraction/evaporation has been used for a long time as a preferred technique for preparation of polymeric micro/nano particulates. It does not require high temperature or potentially toxic phase separation inducing agents. Particles with size ranging from micro to nanometer have been fabricated using this method, though precise control of particle size and size range is still an issue. Polydispersity is often encountered. Also, careful selection of the encapsulation conditions is required in order to achieve a high degree of encapsulation efficiency. Because of the advantages, this technique will be discussed in more detail. Microencapsulation by solvent extraction/evaporation contains four major steps: (a) incorporation of the active compound/drug along with the matrix forming polymer in a liquid solvent phase (frequently organic as dictated by the solubility profile of the matrix forming polymer), (b) emulsification of this first liquid phase into another immiscible liquid phase (frequently aqueous), (c) Extraction of the solvent from the dispersed droplets by the continuum phase accompanied by solvent evaporation, (d) harvesting and drying of the particles.

Incorporation of active ingredient

Lower molecular weight drugs (i.e. molecular weights < 1000 Daltons) have more favorable solubility profile. They are generally of intermediate polarity and can be dissolved in a solvent or solvent system with solvents ranging from acetone to hexane. The biodegradable matrix-forming polymers commonly used have similar solubility

profiles. As a result, for small molecular weight drugs, the incorporation step is relatively easier because both the drug and the matrix forming polymer can be co-dissolved in the same solvent or solvent system.

Unfortunately, for the macromolecular bioactive compounds like proteins this step is immensely complicated by the very different solubility profiles of proteins. Proteins are soluble and have biological activity only in aqueous systems and denature on exposure to most organic solvents especially methylene chloride (Weert, M., 2000; Sah, H., 1999). The protein incorporation problem is thus approached by two different methods; dispersion of small solid micronized protein particles into the solution of the matrix forming polymer; and the dispersion of an aqueous solution of the protein in the organic solution of the matrix forming polymer.

Incorporation of micronized solid protein particles into the matrix forming polymer has been successfully attempted. It is found that encapsulation efficiency is improved with the decrease in size of micronized protein particles (Maa, Y. F., 1997; Al-Azzam, W., 2002). Also spherically shaped protein particles are found to be more efficiently encapsulated than irregular ones (Maa, Y. F., 1997). There is concern about protein stability during the micronization process itself. Unfortunately very little research has been directed towards this problem. Consequently, little literature evidence has been found addressing this concern. Finally, in this technique the size of the drug delivery platform is limited by the size of the solid protein particles.

Proteins are more commonly microencapsulated in the laboratory by water-in-oil-water double emulsion technique. In principle, an aqueous protein solution is emulsified in an organic matrix forming polymer solution and this primary emulsion is again

emulsified in an external aqueous phase. These techniques are complicated and difficult to control and scale up. Generally it is found that increasing the volume fraction of the internal aqueous phase lowers encapsulation efficiency due to droplet coalescence and increased probability of contact between the internal drug solution and the external extraction phase resulting in drug loss (Yang, Y.Y., 2000; Crotts, G., 1995). Increasing the volume fraction of the internal dispersed phase of the first emulsion also results in an increase in particle porosity and burst release (Yang, Y. Y., 2000; Crotis, G., 1995).

Emulsification

Emulsification is the second and probably the most complicated and important step of the particle forming process. Various different techniques have been employed starting from the most rudimentary technique of stirring to increasingly more sophisticated techniques like homogenization with a rotor-stator arrangement, static mixing and extrusion through micro-porous membranes. All of these techniques produce emulsions with different degrees of efficiency, precision and control.

The most important factors that are affected by the emulsification process are encapsulation efficiency, porosity of the particles, denaturation of the macromolecules, particle size, size distribution and reproducibility. These factors affect the release properties of the active ingredients from the particles and thus the overall efficacy and applicability of the platform. The first three factors are affected by the proper choice of polymer, dispersed phase, continuous phase, phase ratio, surfactant and surfactant concentration.

One of the problems of encapsulating macromolecules like proteins is that they often have significant surfactant properties. Most proteins tend to partition to the oil-water

interface to a significant degree as they have both hydrophilic and hydrophobic zones. When drug free microparticles are prepared from emulsions consisting of plain water and PLA dissolved in methylene chloride, increasing amounts of bovine serum albumin (BSA) added to the water as a surfactant stabilizes the emulsion and decreases the pore size of the resulting microspheres. The addition of BSA is thought to be responsible for the surfactant activity, bringing down the water droplet size of the dispersed internal aqueous phase. As the water is extracted, it leaves behind smaller sized pores in the resulting microsphere (Nihant, N., 1994). Also a significant but varied degree of protein loss is observed from the aqueous phase when protein solutions are emulsified with organic phases (Sah, H., 1998). All these observations point to the fact that proteins have varied but significant degrees of surfactant activity. This surfactant activity has a deleterious effect on encapsulation not only because it causes a loss of active ingredient, but also because the denatured proteins sometimes possess altered antigenic behavior Sah, H., 1998). This problem can be addressed to a great extent by introducing competing surface active additives like partially hydrolyzed polyvinyl alcohol (Weert, Marco van de, 2000), hydroxypropyl-β-cyclodextrin (Sah, H., 1998), or a combination of sorbitan esters and polysorbates (Soriano, I., 1995) in the aqueous phase.

Among all the emulsification techniques, the most rudimentary and simplest technique is stirring carried out in a so called continuously stirred tank reactor (CSTR). In this technique the two immiscible phases are forced together by the shearing force of an impeller. The emulsion droplets are formed when the shear force is sufficient and are maintained by the action of surfactants. Homogenization with a rotor-stator arrangement is an advanced version of the simple stirring where the shear force is complemented by

cavitations and thus is more efficient in particle size reduction. Obviously, the most important independent variable that dictates the particle size is the shear force. Dispersed phase viscosity affects the microsphere size by increasing the shear force requirement to bring about comparable size reduction. The increase in continuous phase viscosity results in a decrease in microsphere size as increased viscosity of the continuum phase increases the shear force (for a given rate of shear) acting upon the emulsion droplets.

All available literature points to the simple fact that an increase in the speed of the stirrer/rotor results in decreased microsphere mean size (Yang, Y. Y., 2001; Sansdrap, P., 1993; Gabor, F., 1999; Mateovic, T., 2002). This can be explained by physical mechanics in which increased impellor/rotor speed results in higher shear force, which is the driving force in the size reduction process. The impeller size and shape and the size ratio of the impeller to the mixing vessel also has impact on the microsphere size (Sansdrap, P., 1993), not surprisingly, as these parameters directly affect the shear force.

Increased polymer concentration and thus increased viscosity of the dispersed phase is also known to increase the size of the microspheres produced (Jeyanthi, R., 1997; Marchais, H.,1996; Yang, Y. Y., 2000; Viswanathan, N. B., 1999), as higher viscosity of the dispersed phase also requires higher shear force for size reduction. Increasing the continuum phase viscosity by the addition of a viscosity building stabilizer has exactly the opposite effect on the microsphere size (Capan, Y., 1999; Yang, Y. Y, 2001; Sansdrap, P., 1993; Carrio, A., 1995) because the increase in viscosity also increases the shear force acting upon the dispersed phase droplets at a given shear rate.

The complex interplay of variables involved in determining the microsphere mean diameter in the so called CSTR setup has been mathematically modeled to arrive at an

empirical equation (Maa, Y. F., 1996) relating various factors to particle size. In agreement with previous reports, the model showed strong inverse correlation of the microsphere mean diameter with the increase of stirring speed, impeller diameter (decrease in microsphere diameter) and strong direct correlation with polymer concentration/dispersed phase viscosity (increase in microsphere diameter). It also showed moderate inverse correlation with the increase in continuum phase viscosity (decrease in microsphere diameter) and moderate direct correlation with the increase in interfacial tension (increase in microsphere diameter). The model reproduced and predicted the microsphere diameter with good accuracy for different types of extraction fluids and for microspheres with or without protein loading. However, no prediction regarding the range of particle size distributions and polydispersity could be made.

A relatively newer method of emulsification is through the use of static mixers. Static mixers essentially consist of a series of flow obstacles of baffles in a tube through which the two phase system flows. The baffle arrangement ensures repeated splitting and recombination of the fluid stream and the size reduction occurs mainly by the turbulence created by the baffles. This process has also been empirically modeled (Maa, Y. F., 1996). The correlation with the experimental data as well as the predictive power is found to be very good with the calculated mean diameter deviating from the experimentally determined diameter by less than 10%. A close look into the model equation reveals that increasing the interfacial tension, polymer concentration in the dispersed phase and mixer diameter produce larger microspheres while increasing the flow rate through the static mixer, continuous phase viscosity and the length of the mixer produce smaller microspheres. A comparison of the static mixers with the CSTR system

for emulsification efficiency reveals that the static mixers produce the same degree of mixing at a much lower Reynolds numbers. The uniformity of the particle size of the microspheres is not improved by the use of static mixers. In terms of mixing efficiency and scalability, though, the static mixers have some advantage over the CSTR system (Freitas, S., 2005).

Another more modern method of forming an emulsion is by extrusion. Extrusion in this context means feeding the drug/polymer solution/dispersion through single or a plurality of pathways directly into the continuous extraction phase. Upon leaving the pathway(s), discrete droplets of dispersed phase are formed within the slowly flowing continuous phase, which also transport the droplets from the site of their formation. Extrusion through single pathway systems has been studied for microsphere formation (Leelarasamee, N., 1996). Generally it has been observed that the microsphere size is controlled by needle diameter and the flow rate of the continuous phase at the needle tip, and the adhesion between the forming droplet and the needle tip. The resulting microspheres are large (> 50 micron) and also very poly-disperse with the coefficient of variation for the mean diameter varying from (15-40) %. By increasing the continuous phase velocity, reducing the needle diameter and reducing the adhesion between the needle and the forming droplet with PTFE or silicone coated needles the polydispersity can be narrowed somewhat (Amsden, B. G., 2001). Overall these systems are unsuitable for producing small microspheres (< 50 micron). Multichannel extrusion systems specifically designed for producing mono-disperse particles has been successful in reducing the coeffcient of variation of the mean microsphere diameter to (< 5 %) (Kawakatsu, T., 1999). Still, sub micron sized particles could not be produced, and

achievable throughput in these systems is grossly inadequate for them to be practically useful beyond the laboratory.

Membrane emulsification science has significantly evolved since the advent of a particular glass membrane called Shirasu porous glass and the subsequent observations that highly uniform kerosene-water and water-kerosene emulsions can be made using these membranes (Nakashima, T., 1991). Since then other micro porous membranes of both inorganic and organic origin with pore size ranging from 10 nanometers to 150 microns have been used by researchers for membrane emulsification (Vladislavljevic, G. T., 2005). Products produced by this membrane emulsification through extrusion include metal solder particles, solid lipid and polymeric microspheres, and nonporous and porous core-shell microcapsules to name a few. All types of emulsions including water-in-oil, oil-in-water, water-in-oil-in-water and oil-in-water-in-oil have been reported to be produced. While virtually mono dispersed particles with good control over particle size can be produced by this method, preparing particles in the low sub-micron range is still not reported.

Solvent removal

The dispersed phase solvent needs to be removed for the condensation of solid particles. Solvent removal is generally carried out by evaporation through the use of elevated temperatures, reduced pressure, or by solvent extraction. Apparently, more than the method, the rate of solvent removal affects the performance of the particles produced. Fast solvent removal ensures high encapsulation efficiency by rapid solidification of the matrix material. At the same time, fast solvent removal also results in production of porous particles thereby increasing the release rate. The use of elevated temperatures is

restricted by the thermo labile nature of a number of drugs and proteins. Moderate increase of temperature up to 40°C has been attempted by some researchers resulting in hollow microspheres with porous walls (Jayanthi, R., 1996). Also, there are reports that when the solvent removal is achieved slowly at atmospheric temperature and pressure, there is a tendency to form a crystalline polymer matrix. Solvent removal at reduced pressure produces amorphous polymer matrix (Isumikawa, S., 1991). In an amorphous state, data indicate a molecular dispersion of the polymer and drug which results in the lowering of the release rate of the drug from the microspheres (Freitas, S., 2005). Liquid-liquid extraction is also a widely practiced technique. It is reported that encapsulation efficiency can be significantly improved if a large excess of continuous phase than what is theoretically necessary to extract the dispersed phase is used (Tice, T. R., 1995). The use of tenfold the amount of continuum phase necessary to extract all the dispersed phase solvent is suggested for encapsulating sparingly to freely soluble drug molecules.

2A (*iv*). PRESENCE OF RESIDUAL COMPOUNDS AND RELATED ISSUES

It is imperative that the drug delivery systems intended for the injectable route are nontoxic in addition to being sterile. Even though some polymeric materials are known to be non toxic, biodegradable and biocompatible as required by the Food and Drug Administration (FDA), toxicity can be imparted into the system through the presence of trace amounts of residual surfactant, monomer, polymerization initiators and most importantly, residual solvents. Even if the polymer used is not toxic, it can produce degradation by products that are toxic. One such example is that of nanoparticles composed of poly(cyanoacrylate). While poly(cyanoacrylate) itself is not toxic, one of its

degradation by-products is formaldehyde (Gombutz, W,. R., 1995), which is a known carcinogen (NTP, 81-43, December, 1981).

It has been shown that surfactants can be adsorbed on the surface of the nanoparticles. This adsorption can affect the pharmacokinetic distribution of the nanoparticles (Krueter, J., 1997). As an example, surfactants adsorbed to the surface of butylcyanoacrylate nanoparticles containing the peptide dalargin have been shown to transport them across the blood-brain barrier (Krueter, J., 1997). This also has the potential of changing the therapeutic efficacy of the delivery platform when the presence of residual surfactants on the surface of the particles is unintentional. On the other hand, residual monomers and oligomers present in the particles can act as catalyst in the chain scission reactions thereby causing the premature release of the drug (Niu, C. H., 1998). In the same way, the presence of polymerization initiators, which are generally toxic, is a cause of concern.

Since organic solvents of low polarity have to be used in the fabrication of the nanoparticles, the presence of residual solvents in the final product is a major concern. The most commonly used organic solvent, methylene chloride, is a carcinogen and has been shown to induce other forms of toxicities including neurotoxicity or teratogenicity, especially following inhalation (Kaushik, K., 2004). When mice were treated with 1000 ppm inhaled methylene chloride, increased DNA synthesis and increased cell proliferation in lungs was observed (Foster, J. R., 1994). The USP limits the amount of methylene chloride in pharmaceuticals to 500 ppm. The International Conference on Harmonization guidelines for permissible methylene chloride is 6 mg/day unless it can be shown that the residual solvent is released in a sustained fashion for several days. The effective limit of methylene chloride is 6 mg/dose (Internal Conference on

Harmonization, 1997). Interestingly, with the conventional methods of particle preparation, the residual methylene chloride levels are 3,000-30,000 ppm (Courteille, J. P., 1994; Thoma, K., 1992). The residual methylene chloride can however be reduced to below 25 ppm level in PLGA systems by treatment with supercritical carbon dioxide with a concomitant increase in particle size (about ten fold), decrease in bulk density (five fold) and increase in porosity (Kaushik, K., 2004). Even the presence of residual non toxic solvents can be a problem as they can possibly change the physico-chemical properties of the delivery platform. High residual ethanol levels may reduce the glass transition temperature of PLGA-like polymers, causing the microspheres to aggregate under storage and may result in poor injectability (Sinha, V. R., 2003). Consequently, careful design and selection of the manufacturing procedure, selection of the materials and solvents are essential for the success of the delivery platform.

2B. PHYSIOLOGICAL RESPONSE TO PARTICULATE DDS

Once the nanoparticulate drug delivery systems are introduced into the systemic circulation, two major events take place; (a) the particles are recognized by the cell mediated immune system of the body and promptly eliminated by phagocytosis, (b) the surviving particles are subsequently taken up by the cells by endocytosis in varying degrees depending on their size and surface properties. Phagocytotic removal of the particulates is undesirable for drug delivery and/or targeting especially with respect to macromolecular drugs for a variety of reasons.

Most proteins including the physiologically active ones have a very short biological half life. Even if these proteins can be released slowly from the particulate DDS injected

intramuscularly or subcutaneously, the problem of short half life of these molecules are not solved. Additionally, the uptake of these released proteins into the cells, the most likely site of their action, remains a problem. DNA or genes also are not taken up appreciably by cells. That is why, except for very exceptional cases, successful drug delivery systems for macromolecules require delivery of the bioactive molecules to their site of actions, i.e., into the cells. For successful cellular delivery and targeting, the first and foremost condition to be met is that the delivery device has to remain in systemic circulation for a sufficiently long time. Phagocytotic removal of the traditional drug delivery systems from systemic circulation is thus a serious limiting factor.

The complex cascade of events following the introduction of foreign particles into the systemic circulation that leads to their rapid clearance from systemic circulation are better understood in recent years. Conventional colloidal drug carriers are rapidly cleared from the blood stream by the mononuclear phagocyte system, mainly represented by the Kupffer cells of the liver and spleen macrophages (Stolnik, S., 1995). This removal from the circulation generally occurs through specific recognition by cellular receptors specific for the plasma proteins bound to the carriers rather than the carriers themselves. The deposition of the plasma proteins on the surface of the carriers is dependent on the surface charge, size, hydrophilicity/hydrophobicity and the conformation of the polymer chains among others.

It is now widely accepted that the opsonization process starts with the activation of complement systems, most importantly the C3 system (Artursson, P., 1986; Kazatchkine, M. D., 1988; Brazile, D., 1995). The activation of C3 complement results in proteolytic degradation of the C3 molecule producing two fragments C3b and iC3b (Kazatchkine,M.

D., 1988; Brazile, D., 1995; Jensen, J., 1996). C3 products can be generated by the classical or alternative pathways of complement activation, requiring Ca^{2+} and Mg^{2+} ions. The activation of alternative pathway is regulated by the nature of particle surface. The cleavage of C3 by C3 convertase is induced by the functional groups at the surface of the foreign particles and results in the deposition of C3b onto them. Also the surface of nanoparticles prepared from biodegradable polyesters, especially PLA, act as activators of the complement system (Mosqueira, V. C. F., 2001). The macrophages are equipped with two receptors namely CR1 and CR3 which recognize the complement fragments C3b and iC3b. Thus, following the complement activation, the complement fragments generated by the proteolysis are adsorbed onto the surface of the particles which are then phagocytosed by the macrophages through the complement fragment recognition receptors present on their surface (Mosqueira, V. C. F., 2001 & 1999). Since the activation of the complement system by the alternative pathway is influenced by the nature of the particle surface, efforts are made to modify the particle surface in a manner that the complement activation, if any, is minimized (Gref, R., 2000; Vittaz, M., 1996). It has been found that the plasma half life of PLA nanoparticles is two to three minutes. When they are coated with either human serum albumin or pluronic surfactant F68 (polyoxyethylene-polyoxypropylene) does not increase the plasma half life. But when polyethylene glycol (PEG) is used to modify the surface, the resulting PLA-PEG nanoparticles showed an increased half life of six hours (Verrecchia, T., 1995). Further, it is found that the density of PEG chains grafted to the surface has a reciprocal relationship with the C3 cleavage. The higher the PEG density, the lower is the C3 cleavage. Increasing the PEG chain length decreases the C3 cleavage even at a lower

density (Mosqueira, V. C. F., 2001). The longer chains, at the same surface density, were hypothesized to cover the surface better with the so called brush or mushroom configuration of the chains. Overall, the hydrophobic surface, mostly dictated by the choice of the polymers, of the nanoparticles seems to be a major limitation towards development of long circulating particulate delivery systems.

Endocytotic uptake of the particulates by the non-immune cells, on the other hand, can be helpful. If sufficient control and selectivity can be achieved through suitable modifications of the surface of the particulates, endocytosis along with receptor mediated uptake can be an effective tool in delivering the active payload by the particulates into the target cell.

It has been known for about one hundred years that intact micro and nanoparticles of natural origin can be absorbed across the gut wall at least to some extent. Raw starch granules fed to rats have been known to be absorbed across intestinal mucosa for a long time (Verzar, F., 1936). More recently, a wide variety of particles starting from titanium dioxide, to polyvinylpyrrolidine-coated silica (Florence, A. T., 1997), and polystyrene nanoparticles (Jani, P., 1989) have been shown *in vivo* experiments to translocate across the gastro-intestinal tract lining. The extent of absorption following oral administration though is believed to be quite small (2-3% of the administered dose) (Florence, A. T., 1997).

Efforts have been made to characterize the cellular uptake of particles *in vitro*. In most cases, cultured epithelial cells of various origin including Caco-2 representing intestinal mucosa, human umbilical vein endothelial cells (HUVEC), African green monkey kidney endothelial cells (COS-7) and human embryonic kidney epithelial cells

(HEK-293) have been used. Results of all these investigations have consistently suggested a few key features. Firstly, rapid uptake of nanoparticles by endothelial cells has been demonstrated (Davda, J., 2002). Further the uptake is found to be dependent on the concentration of the nanoparticles. The particles are found to be localized into cytoplasm. No adverse side effects have been detected in the cells as a result of the nanoparticle uptake, as shown by mitogenic assay 48 hours following the particle intake. Secondly, the uptake has been shown to depend on the temperature of incubation with relatively lower uptake in 4°C than in 37°C. This suggests that the process is energy dependent and is mediated by endocytosis (Quaddoumi, M. G., 2000). Further, it is found that the endocytotic uptake of the nanoparticles is size dependent (Prabha, S., 2002; Desai, M.P., 1997). The efficiency of cellular uptake in Caco-2 cells varied from 41% for 100 nm particles to 15% for 1 micron particles and 6% for 10 micron particles. Similarly, when PLGA nanoparticles containing plasmid DNA encoding luciferase protein is used for gene transfection studies, smaller particles with mean diameter of 70 nm showed 27 fold higher transfection than larger sized nanoparticles with mean diameter of 202 nm in the COS-7 cell line and a 4 fold higher transfection in the HEK-293 cell lines (Prabha, S., 2002).

In an effort to understand the process of internalization of the nanoparticles better, it has been hypothized that the internalization is a two step process. The first step is the adsorption of the particles onto the plasma membrane of the cell surface. The second step is endocytosis. Both processes occur concomitantly at 37°C, whereas only the first step occurs at 4°C. When anionic maghemite nanoparticles are incubated with human ovarian tumor cells (HeLa) and a cultured mouse macrophage cell line (RAW-264.7) at

4°C for one hour (endocytosis inhibited), the adsorption of the nanoparticles on the outer cell surface is found to be saturable both in terms of time of incubation and extra cellular concentration of the nanoparticles (Wilhelm, C., 2003). When, the same nanoparticle cell system is then incubated at 37°C for one hour, again the internalization process showed saturable kinetics for both the cell types in terms of time and extra cellular nanoparticle concentration. In both cases, the quantifications are done by two different methods based on two different physical properties namely magnetophoresis (MP) and electron spin resonance (ESP). Measurements from both the methods showed remarkable agreement. Visualization of the whole process has also been attempted in stages using a transmission electron microscope (TEM). TEM pictures of the HeLa cells fixed after one hour incubation at 4°C (endocytosis inhibited), showed the adhesion of anionic nanoparticles on the plasma membrane mainly in the form of clusters probably through electrostatic interactions. TEM photographs were also taken of the cells after the same incubation at 4°C for an hour but followed by ten minutes and an hour of incubation at 37°C (restoring endocytotic activity). After ten minutes of incubation at 37°C, TEM photographs showed early events of cell internalization of calthrin coated vesicles containing nanoparticles. Following an hour of endocytotic activity, densely confined nanoparticles into endocytic organelles with various morphological features and cytoplasmic localizations including late endosomes and lysosomes with micromeridic sizes could be identified. This is by far the most comprehensive proof of endocytosis of nanoparticles by cells found by this author in contemporary literature. Solid lipid nanoparticles containing spin labeled compounds have also been found to be taken up by leucocytes by endocytosis (Kristl, J., 2003).

Recently, efforts have been made in targeting nanoparticles to different target cells. A monoclonal antibody (MAb) with specificity for rabbit M cells was attached covalently to one micron polystyrene particles and investigated for cellular uptake. The MAb indeed increased the particle count in rabbit Payer's patches whereas a nonspecific MAb had no effect. Similarly, tomato-lectin bound to microspheres induced a marked increase in systemic uptake, which could only be accounted for by induction of uptake through enterocytes as well as lymphoid tissue. Tomato-lectin coupled microspheres showed unusual absorption tendencies. Single dosing of animals produced no evidence of uptake, whereas five to ten days of daily dosing caused uptake of 18% of the dose (Florence, A. T., 1996), probably due to the induction of receptor expression by lectin. Interestingly lectins from different sources are known to bind to specific cells in different regions of the gastro intestinal tract. Though the last few examples show systemic absorption, they are sited because the systemic absorption of the particulates occurs in these cases through activation and targeting of a particular cell type in the gastro intestinal tract.

3. CURRENTLY AVAILABLE PARTICULATE PLATFORMS

A plethora of different particulate polymeric platforms are currently available for drug delivery purposes. These platforms mainly differ in the physical and chemical properties of their constituent polymers and also the change in methodology dictated by the polymers.

3A. HYDROPHOBIC SYSTEMS

3A (*i*) BULK EROSION POLYMERIC SYSTEMS

PLA, PLGA AND ASSOCIATED SYSTEMS

The most successful and most widely investigated particulate platforms are made up of polymers of either lactic acid or both lactic acid and glycolic acid. The main advantage of this class of polymers is their well known biodegradability and biocompatibility (Visscher, G. E., 1985, 1987; Yamaguchi, K., 1993). Due to decades of use of these polymers as medical sutures, toxicological, chemical, and bio/histocompatability data are readily available. Other attractive features of this class of polymers include predictable biodegradation kinetics, commercial availability and most importantly the relative ease of regulatory approval (Lewis, D. H., 1990).

Particles composed of PLGA and PLA undergo homogeneous bulk erosion on being exposed to water or physiological fluid (Gliding, D. K., 1981). Initially there is random cleavage of hydrogen bonds due to hydration followed by cleavage of ester bonds leading to a decrease in molecular weight of the polymer and production of lactic and glycolic acids. These are metabolized in the body through the Kreb's cycle and eliminated as carbon dioxide and water. The carrier retains its original shape until significant degradation has occurred (~90%) (Sinha, V. R., 2003). It dissolves completely once the molecular weight is reduced to the extent that it becomes soluble. The chemical composition and ratio of the monomers used in the polycondensation reaction strongly influence the degradation and thus the release kinetics as well. The degradation kinetics is also influenced by polymer chain packing (crystallinity), hydrophobicity and

sterioregularity. Since some of these parameters like molecular weight, molar ratio between lactic and glycolic acid monomers, stereo regularity, and crystallinity can be easily controlled at least partially, resulting polymers with varying degradation time and release properties are readily available for use in the particulate DDS.

While the obvious advantages of this class of polymers encouraged a spurt in research activity as sustained release particulate DDS, their success in terms of macromolecular delivery is rather limited. Except Nutropin Depot which delivers growth hormone, all such products deliver small molecules including three leutenizing hormone releasing hormone analogs (decapeptide or less) and all are either intra-muscular or subcutaneous injections, or implantable rods (Tice, T, 2004). A long circulating reservoir type particulate formulation for sustained release of the macromolecule in the systemic circulation is still not achievable with the current level of sophistication in the associated technology.

PLA and PLGA particulate products suffer from several limitations. Their hydrophobicity and solubility profiles dictate the choice of solvents ranging from acetone to dichloromethane. The removal of organic solvents from PLA, PLGA and most other hydrophobic polymers is problematic at best. With conventional methods of particle preparation, the residual organic solvent levels can be as high as 3,000-30,000 ppm while the USP limit is in the vicinity of 500 ppm. The residual organic solvents can be removed by supercritical carbon dioxide processing, but the resulting particles become very porous with around a ten fold increase in size (Kaushik, K., 2004). Most biological macromolecules are not soluble in these solvents, rather they tend to precipitate and aggregate often irreversibly in their presence (Carpenter, J. F., 1989; Sah, H., 1999). The

incorporation of macromolecules into these particles is thus complicated. It is generally done in the form of solid micronized macromolecule crystals suspended in the polymer solution or dissolving the macromolecule in the aqueous phase and emulsifying the aqueous phase in the polymer solution (Hermann, J., 1998). Either of these methods results in an increase in particle size making them more vulnerable to phagocytosis. The hydrophobic nature of these polymers often cause multilayer adsorption of the protein on the polymer surface (Horbett, T. A., 1987; Verrecchia, T., 1993) often resulting in incomplete release. Also these polymers produce the monomeric acids as the particles degrade in the body, producing a very acidic microenvironment which is not conducive to the stability of a number of macromolecules.

The most serious limitation of the PLA and PLGA systems is the activation of the cell mediated immune system caused by introduction into the systemic circulation. The plasma half life of PLA and PLGA nanoparticles is around two to three minutes. To minimize this problem a new class of polymers has been introduced: PLA-PEG and PLGA-PEG which are polyethylene glycol covalently attached to PLA or PLGA chains. In the particles prepared from these polymers through similar methodology, the hydrophilic PEG chain orients itself appropriately in aqueous suspension such that the more hydrophobic PLA and/or PLGA surface is shielded from the aqueous interface. With appropriate choice of molecular weight and PEG density on the surface it has been possible to reduce the immune system activation resulting in an increased plasma half life from two to three minutes to six hours (Mosquerra, V. C. F., 2001; Verrecchia, T., 1995). Though this is a significant improvement, clearly this is not adequate for systemic delivery. Perhaps in recognition of this limitation and potential of the activation of the

immune system, PLA and PLGA micro/nanosphere research is now focusing more and more on vaccine delivery (Jiang, W. L., 2005; Bramwell, V. W., 2005; Waeckerle-Men, Y., 2005).

POLYACRYLATES

Poly(acrylate) or more appropriately poly(alkylcyanoacrylate)s are another group of very well explored polymers for particulate drug delivery platforms. The main attraction of poly(alkylcyanoacrylate)s is the ease of manufacturing of the particles by polymerization simultaneously as the particles are formed without the need of toxic initiation catalysts. They undergo spontaneous polymerization at room temperature when the monomers are dissolved in water and a hydrophobic interface is provided in the form of an organic solvent or when oil is emulsified in the continuum phase. As the polymerization progresses, the molecular weight increases and the polymer appears in the organic phase in the form of porous particles.

The drug can be loaded by introducing the molecule in the continuous phase at the beginning or the end of the reaction (Couvreur, P., 1979; Bech, P. H., 1993, 1994). The introduction of the active ingredient at the beginning of the reaction results in its incorporation in the core (Bech, P. H., 1993, 1994), while introduction of the active ingredient at the end of the reaction results in its adsorption at the surface (Losa, C., 1991; Bech, P. H., 1993). The whole incorporation process seems to be adsorption controlled. A number of different molecules like the antibiotic gentamicin ((Zang, Q., 1998), human immunodeficiency antiretroviral agent stavudine (Kuo, Y. C., 2005), analgesic peptide dalargin (Kreuter, J., 1997) and proteins like insulin (Zang, Q., 2001;

Sullivan, C. O., 2004) have been successfully incorporated into poly(alkylcyanoacrylate) nanoparticles.

Long term stability of polybutylcyanoacrylate nanoparticles has been studied in suspensions in various aqueous based media (Sommerfeld, P., 1997) including dilute hydrochloric acid solution, distilled water, phosphate buffered saline and human serum. It has been found that the particles tend to agglomerate during separation and recovery (Kreuter, J., 1983; Sommerfeld, P., 1997). An increase in both polydispersity and particle size occurred following each step. This demonstrates that the final size of the particles is heavily dependent on the recovery process and reproducibility is difficult to achieve. Further, purified nanoparticles showed rapid degradation in aqueous suspension accompanied by particle size reduction and significant aggregation. The degradation process accelerated at higher temperature. When suspended in phosphate buffered saline the nanoparticles aggregated rapidly with sedimentation. However the particle suspension could be preserved in dilute (0.01N) hydrochloric acid solution for over two months. Also, the particle suspension is found to be very stable with no aggregation in human serum for five days with significant aggregation appearing only after eight days.

Polyalkylcyanoacrylates are not toxic as a class of polymers. However they show significant toxicity *in vivo* because of the toxic degradation by products. Acrylic acid polymers are bulk erosion polymers undergoing random chain scission until they become soluble with the formation of the toxic by product formaldehyde. This reaction is strongly dependent on the polymer molecular weight and the pH of the medium (Leonard, F., 1966). The alkylcyanoacrylates also undergo the same reaction along with a parallel side chain scission by the blood esterases producing the corresponding alkanol

(Lenaerts, V., 1994). Comparative *in vitro* cytotoxicity studies have been carried out to assess the relative toxicity of different polymers including polyalkylcyanoacrylates and PLGA (Zange, R., 1997). The conclusion of the study is that all alkylcyanoacrylates are more toxic than PLGA and generally the toxicity increases with the decrease of the size of the alkyl side chain. The polymer concentration level at which the cytotoxicity is observed gives the following increasing order of toxicity starting from the lowest.

PLGA > BCA > PVC > HCA > ECA > MCA

In addition to toxicity, the polyalkylcyanoacrylate nanoparticles suffer from the same disadvantage as the PLGA nanoparticles in that they are instantaneously detected by the reticuloendothelial system of the host body and are promptly eliminated from the blood stream (Grislain, L., 1983; Lenaerts, V., 1984; Gibaud, S., 1996). They accumulate in the liver, spleen and bone marrow. As a consequence these nanoparticles are unsuitable as prolonged delivery platform to be used in systemic circulation.

3A (*ii*) SURFACE EROSION SYSTEMS

POLYANHYDRIDES

Polyanhydrides represent a new class of biodegradable polymers meeting some of the essential requirements of a sustained release particulate drug delivery system. They are composed of highly water-labile anhydride bonds joining dicarboxylic acids of different molecular structures and weights in series to make up the polymer molecule. The polyanhydrides that are important from drug delivery purposes are all made up of bulky or hydrophobic dicarboxylic acids. The hydrophobicity of the monomeric dicarboxylic acids decreases the rate of penetration of water into the polymeric device. Since the

anhydride bonds are highly water-labile, on being exposed to water the polymeric particles erode from the surface (outside in) as water slowly penetrates into them. A continuous decrease in device thickness throughout the erosion process, maintenance of structural integrity and nearly zero order degradation kinetics suggest dominancy of heterogeneous surface erosion (Angelova, N., 1999). As the polyanhydrides can be synthesized from a wide range of monomers with varying polarity, their degradation rate can be controlled over a wide period of time with over several thousand fold difference in rate (Domb, A. J., 1987, 1989). The aliphatic polyanhydrides erode very fast and thus are not very useful for pharmaceutical applications. The aromatic polyanhydrides degrade extremely slowly (Domb, A. J., 1992). Thus the aromatic and aliphatic copolymers are best suited for drug delivery applications with optimum degradation profiles and mechanical properties. The hydrolysis of the anhydride bonds are base catalyzed and the degradation of the polymer is dependent on pH and solubility of the degradation products in the surrounding media. Thus the degradation is slower in acidic pH at which the monomers are unionized and thus have limited solubility (Jain, J. P., 2005).

Toxicity associated with polyanhydrides has been investigated in terms of cytotoxicity, mutagenicity and local irritation. For cytotoxicity and mutagenicity studies, cultured bovine endothelial and bovine smooth muscle cell models, which are very sensitive to the changes in growth media and substrate (Leong, K. W., 1986), have been used. The study showed an absence of acute toxicity of these polymers or their degradation products to sensitive mammalian cells. Since carcinogenesis is usually preceded by mutagenesis; mutagenicity testing has been used for rapid screening of neoplastic transformation. The *in vitro* results show that polyanhydrides, particularly the

more popular poly[1,3-bis-(p-carboxy)-poly[1,3-bis(p-carboxyphenoxy)methane]-co-sebasic anhydride] [P(CPP-SA)] are non cytotoxic and non mutagenic but have a very low teratogenic potential (Leong, K. W., 1986). *In vivo* studies with poly[1,3-bis–(carboxyphenoxy)propane] and poly(terephthalic -sebasic anhydride) by implantation of polymer samples into the cornea of rabbits has shown no indications of local irritation like loss of corneal clarity, inflammation or proliferation of new blood vessels (Leong, K. W., 1986) for six weeks. Similar experiments involving subcutaneous insertion of polymer disks in rat models for eight weeks failed to detect any systemic toxicity (Laurencin, C., 1990). It is to be noted that no long term carcinogenicity studies on polyanhydrides has been reported to date (Jain, J. P., 2005).

Polyanhydrides are primarily developed for injection molding, compression molding and hot-melt extrusion. The last two processes require elevated temperature and are thus not well suited for thermo labile compounds like proteins (Leong, K., 1986). Polyanhydrides had also been reportedly used to develop particulate delivery system containing proteins like lysozyme, trypsin, heparinase, immunoglobulin (Tabata, Y., 1993) and bovine serum albumin (Determan, A. S., 2004) using double emulsion technique. They have been more extensively studied for various applications as localized delivery carriers (Jain, J. P., 2005) for various disease conditions including cancer (Teomim, A., 1999; Krasco, M. Y., 2003; Li, Y., 2004), glaucoma (Uppal, P., 1994), and Alzheimer's disease (Howard III, M. A., 1989). Recently, one of the polymers in this class, namely [P(CPP-SA)], has been approved by the FDA for local delivery in the treatment of brain cancer (Wang, P. P., 2002). This is one of the few examples where a biodegradable synthetic polymeric implant has been approved for human use by the

FDA. Generally speaking, most of the drug delivery research is specifically focused on local delivery matrices like implants, films, and surgical pastes mostly involving highly hydrophobic chemotherapeutic agents and other hydrophobic drugs.

In an overall analysis, polyanhydrides do not seem to be drastically different from the more established platforms like PLGA. Being hydrophobic polymers, particles have to be formulated with hydrophobic organic solvents in the internal phase. So the problems like macromolecule denaturation, presence of residual organic solvents and aggregation associated with the nature of the polymers and size associated with the necessary double emulsion technique still remain. Additionally and most importantly, polyanhydrides being a more hydrophobic class of polymers are expected to activate the cell mediated immune system more strongly and thus a free circulating particulate injectable formulation does not seem to be a possibility. No study showing phagocytosis of polyanhydrides, or the lack of it, could be found in literature. Probably because of these factors the focus of research involving this platform is more on local delivery of hydrophobic small molecules like chemotherapeutic agents. Lastly, long term safety of the polyanhydrides is still to be established.

3B. HYDROPHILIC SYSTEMS

A wide variety of hydrophilic polymers have been investigated for drug delivery purposes. Synthetic polymers like poly(ethylene oxide) and poly(propylene oxide) and their copolymers, polyvinyl alcohol, polyvinyl pyrrolidone as well as natural polymers like starch, dextran, polyanionic carbohydrates like alginic acid, polycationic carbohydrates like chitosan and proteins like albumin, gelatin, and collagen have all been tried for this purpose and have shown some promise for restricted applications. One

common advantage of the hydrophilic polymer based platforms is that they are all aqueous based and thus represent a much more benign microenvironment for the bioactive macromolecules like proteins. Secondly, being hydrophilic they show a much reduced complement activation and the resulting immune recognition. Some of the more successful and promising platforms will be considered in more detail here.

ALGINATES

Alginates are linear polysaccharides containing varying amounts of 1, 4'-linked β-D-mannuronic acid and α-L-guluronic acid residues. These two acids are arranged in random domains of homopolymer blocks of one kind and the domains do not follow any particular pattern. The exact proportion of guluronic acid and mannuronic acid residues vary in alginates according to its natural source with some alginates rich in guluronic acids while others rich in mannuronic acid (Huang, A., 1962). The molecular weight of the alginates vary widely depending on its source from 80-290 kilo Daltons and the viscosity of alginate solution increases with increasing molecular weight (Berth, G., 1992). Two special properties sustained the interest in alginates for drug delivery; poly ion complexation and resulting gelation of alginates when in contact with polyvalent metal ions or cationic polymers in solution and the mucoadhesive nature of the polymer. The mucoadhesion of alginates to living intestinal epithelial lining of rat has been investigated and tensile strength quantified (Chickering, D., 1992, 1995). It is found that alginate has the highest mucoadhesive strength when compared to polymers such as polystyrene, chitosan, carboxymethylcellulose and poly(lactic acid).

A variety of different proteins have been incorporated into alginate micro/nano particles like antigens (Bowersock, T. L., 1996). Most of the work, though, seems to be directed towards local delivery of growth factors like fibroblast growth factor (Edleman, E. R., 1993) for tissue repair, nerve growth factor (Maysinger, D., 1992) for treatment of age dependent neuronal atrophy of fore brain and anticancer proteins like tumor necrosis factor receptor and interleukin receptors like interleukin-17, 1, 4 receptors (Gombotz, W. R., 1998). In addition, alginate gelation has been successfully used to encapsulate and preserve live cells like chromaffin cells (Tsang, P. W., 1996), and islet cells (Lim, F., 1980), etc., with the overall goal of hormone or neurotransmitter replacement therapy through cell transplantation. It is observed that basic proteins show more prolonged release patterns than acidic proteins when encapsulated in alginate particles due to obvious ionic interactions between the polymer and the protein in the first case, and the lack of it in the later. Also, basic proteins, like basic fibroblast growth factor is found to be largely deactivated due to the ionic interactions with alginates (Mumper, R. J., 1994).

Alginate micro/nano particles are prepared by poly ion complexation of sodium alginate solution with divalent metal ions like Ca^{2+}, Ba^{2+}, Sr^{2+} (Gombotz, W. R., 1998) and Al^{3+} (Al-Musa, 1999). Monovalent cations and Mg^{2+} do not induce gelation. The use of other divalent cations such as Pb^{2+}, Cu^{2+}, Cd^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+} and Mn^{2+} causes gelation but their use is limited by their potential toxicity. The gelation and crosslinking mainly occur by the exchange of sodium ions from guluronic acid residues with the divalent cations and the stacking of these guluronic acid groups of two different polymeric chains together in a highly cooperative manner. The size of the cooperative units is more than twenty monomer units (Smidsrod, O., 1990). It is known that the

crosslinking reaction is fast and the reaction is complete within thirty minutes. The tightness of crosslinking and thickness of the crosslinking layer is dependent on the ionic radius of the polyvalent metal ion (Al-Musa, 1999). Calcium and barium ions having greater atomic diameters of 0.95 Å and 1.35 Å, respectively, produce looser crosslinking of lesser thickness. Aluminum ion with an atomic radius of only 0.5Å produces tighter crosslinking throughout the matrix. The resultant *in vitro* release profile also verifies these observations by showing varying rates of diffusional release.

Since the alginate particulate platform is held together by ionic interactions, change in ionic environment and pH affects its stability and structural integrity. The alginate gel structure breaks down by removal of polyvalent metal ions from the environment using chelating agents. High concentrations of anions like lactate, citrate and phosphate break down the gel structure. A high concentration of cations like Na^+ and Mg^{2+} in the medium also cause a breakdown of the gel structure due to formation of more preferred complexation species and breakdown of metal ion-guluronic acid complex (Sutherland, I. W., 1991). Alginates are protonated in acidic medium producing alginic acid and breaking down the gel structure (Al-Musa, 1999). The alginates are also hydrolyzed in acidic media leading to a decrease in molecular weight and faster release of the encapsulated molecules (Huang, A., 1963). This acid catalyzed degradation and breakdown of poly ion complexation limits the use of monolithic alginate particles for oral delivery of drug molecules. The mucoadhesive properties of alginic acid can be utilized if the alginic acid is used in particle formation along with other biodegradable polymers.

Though the alginates are found to be largely biocompatible, they do produce an immunogenic response. There are numerous reports of alginates producing fibrotic reaction (Soon-Sheong, P., 1991; Otterlei, M., 1991; Spargo, B. J., 1994). The immunogenic reactions of alginates seem to be related to its chemical composition and mitogenic contaminants (Zimmerman, U., 1995). Though all kind of alginates activate cytokine production upon the introduction of alginate particles into the blood stream, alginates rich in mannuronic acid activate cytokine production more than alginates rich in guluronic acid (Soon-Sheong, P., 1991; Otterlei, M., 1991; Spargo, B. J., 1994). Irregular surfaces of the particles also contribute to the fibrotic reaction of the alginates (Vos, P.D., 1996).

Overall, the alginates appear to be a promising platform for sustained release of macromolecules because of the benign microenvironment and relative biocompatibility. *In vitro* studies also show sustained release for large macromolecules. The immunogenic reactions caused by the alginates remain a concern and the phagocytotic reaction to alginates has not been investigated in depth. Also, alginate hydrogel stability *in vivo* is suspect due to the high ionic strength, presence of enzymes and presence of specific ionic species known to affect the stability of the hydrogel structure. No sustained release alginate product has been approved so far after more than two decades of research in this field.

GELATIN

Gelatin is a natural polymer derived from collagen and is commonly used in pharmaceutical and medical applications due to its biodegradability (Balakrishanan, B., 2005; Yamamoto, M., 2001) and biocompatibility in the physiological environment (Yao,

C.H., 2004). Gelatin has a proven record of safety as a plasma expander and as an ingredient in drug formulations and as a sealant for vascular prosthesis (Kuijpers, A. J., 2000). Collagen can be pretreated (before extraction) with acid or alkali to produce basic gelatin with an isoelectric point (IEP) of 9 or acidic gelatin with IEP of 5 (Ikada, Y., 1998). The difference in IEP is because of the fact that acid treated gelatin possesses more amino groups than alkali treated gelatin which possesses more carboxylic acid groups. As a result gelatin is a good polymer for polyion complexation either between two of its own species to produce a electrovalently bonded structure or with macromolecules of different overall charge to increase encapsulation efficiency and decrease release rate.

Because of the good gelation properties, gelatin has been extensively investigated for local sustained release applications in the form of block hydrogels and films. Although polyion complexation is important in the context of biomolecule loading and retention, the key determinant of controlled release from these constructs is the rate of degradation of gelatin carriers (Yang, S., 2005). Given that gelatin gel undergoes rapid degradation *in vivo* whereas chemically crosslinked gelatin gels are relatively stable, most gelatin-based platforms relevant to sustained biomolecule delivery use chemical crosslinking. *In vivo* models have been used to assess the effect of crosslinking density on the rate of gelatin degradation (Tabata, Y., 1999). Gelatin hydrogels are chemically crosslinked by using aldehydes like formaldehyde, glyceraldehyde and gluteraldehyde, polyepoxides and isocyanates (Kuijpers, A. J., 2000). The carboxylic and amino groups of gelatin can also be directly crosslinked by using chemical activators like acyl azides and carbodiimides (Tabata, Y., 1994). As expected, crosslinking density in gelatin gels is found to be

inversely proportional to hydrated water content of the gel and as also the rate of gel degradation *in vivo* (Tabata, Y., 1999).

Different growth factors have been incorporated into gelatin hydrogels for hard tissue regeneration (Yamamato, M., 2003), soft tissue regeneration (Kimura, Y., 2003) and therapeutic angiogenesis (Thomson, J. A., 1988) with pharmacodynamic improvement noted in each case for a prolonged period of time. Gelatin films have been used for local delivery of insulin from open wounds (Shinde, B. G., 1992) and also in local delivery of basic fibroblast growth factor to the heart following artificial acute myocardial infraction in experimental animal models for neovascularization in the heart (Ueyama, K., 2004). Crosslinked gelatin microparticles have also been investigated for sustained local delivery of mainly growth factors (Kimura, Y., 2003; Shakakibara, K., 2003). These investigations showed marked pharmacodynamic effect in both cases.

The most important drawback of gelatin based drug delivery platform is that polyion complexation by gelatin is not enough to produce significant sustained release by itself. The crosslinking techniques used to slow down gelatin degradation are too generic and result in crosslinking in the bulk of the polymer. Such crosslinking in the bulk can react with the bioactive macromolecule resulting in deactivation (Gombotz, W. R., 1995). Additionally, gelatin particles have not been reported in the submicron range in size thereby making it unsuitable as a systemic delivery vehicle. Indeed not a single investigation has been found aimed at using the gelatin particulate platform for long circulating systemic and sustained release. Though gelatin is a hydrophilic polymer and has been used as plasma expander, it does produce activation of RES in particulate form. Gelatin is known to adsorb fibronectin, a blood component that provides particle

recognition (Tabata, Y., 1989). In totality, gelatin though largely biocompatible and which can effect polyion complexation, is not very useful as a particulate delivery platform for sustained release systemic delivery of bioactive macromolecules.

STARCH, DEXTRAN AND ASSOCIATED SYSTEMS

Starch, dextran and associated natural polymers and their derivatives have been investigated as a sustained release particulate delivery platform for bioactive macromolecules for a long time. The use of biodegradable hydrogels for delivery of bioactive macromolecules like proteins is of particular interest due to their biocompatibility and their relative inertness towards protein drugs (Park, K., 1993), (Domb, A., 1990). Hydrogels are the only class of polymeric systems that allow proteins to permeate through the continuum of the carrier (Gombotz, W.R., 1995). The hydrogels rapidly swell with exposure to water and can rapidly decrease in mechanical strength, and often integrity, if they are not reinforced with chemical crosslinking to control swelling. The initial rate of release of macromolecules from hydrogel systems are diffusion controlled through the aqueous channels of the gel structure. Generally the rate is inversely proportional to the molecular weight of the encapsulated molecule and directly proportional to the equilibrium water content characteristic of the gel.

Crosslinked starch (Degling, L., 1993), dextran (Kim, S. H., 1999), amylopectin (Michailova, V., 2001) and cellulose derivative (Bech, S. L., 1990) based hydrogels have all been investigated for this purpose. It has been generally found that carbohydrate based hydrogel systems release drug molecules in a few hours (Michailova, V., 2001). The drug release roughly corresponds to the time that is required for the hydrogel system to attain equilibrium swelling. In an effort to reinforce the hydrogel structure a wide

variety of chemical crosslinking methods have been tried. Polyfunctional aliphatic acids (Sediel, C., 2001), epichlorohydrin, phosphorous oxychloride, methylenebisacrylamide (Denizli, B.K., 2004) have all been employed to modify starch and dextran to improve the mechanical properties and decrease swelling of the resulting hydrogels. Epichlorohydrin crosslinked dextran hydrogel shows the minimum equilibrium swelling of 900% while phosphorous oxychloride crosslinked dextran shows the maximum equilibrium swelling of 5450%. Dextran has also been derivatized with the sodium salt of acrylic acid to produce dextran acrylate. The acrylated dextran gel was then photo crosslinked by long wave UV irradiation. The resulting product showed relatively lower equilibrium swelling of 171% at pH 7 (Kim, S. H., 1999). Polyacryl starch (Artursson, P., 1984) and polyacryl dextran (Edman, P., 1980) microspheres have been investigated for immobilization and delivery of several proteins and also liposomes (Stenekes, R.J.H., 2000).

Though these systems have shown some promise as far back as the early 1980s, the problems associated with rapid swelling, fast release of the contents or incomplete release of proteins could not be solved satisfactorily until now. Additionally, all these systems suffer from the basic disadvantage in that they are crosslinking in the bulk of the polymer matrix. This kind of bulk crosslinking can denature the incorporated macromolecule or covalently incorporate the macromolecule in the polymer matrix and thus make the macromolecule unavailable for release.

4. CONCLUSION: NEED FOR A NEW APPROACH

Certain general conclusions can be made from this review of theavailable literature. To be effective and clinically useful, long circulating, sustained release, particulate delivery systems for bioactive molecules, especially macromolecules, have to fulfill certain essential criteria. In addition to being biodegradable and biocompatible and non toxic, they have to be able to protect the stability of the incorporated bioactive macromolecule throughout storage and during the actual use. In this respect, processing parameters like the use of organic solvents, and the presence of trace amount of residual solvents and their effects on the incorporated macromolecule, is as important as the safety and biocompatibility of the polymer used. The systems must not show irreversible interactions like adsorption to the incorporated bioactive molecules, especially proteins. The system should be transparent to the immune system and not produce appreciable complement activation.

It is recognized that no current platform completely fulfills all the necessary criteria. The more successful hydrophobic platforms like PLGA and associated systems have the advantage of better control of the release rate of the incorporated molecules. But they suffer from the disadvantage of greatly activating the immune system and thus are not suitable for systemically circulating particles. Interestingly, not only PLGA, but all such hydrophobic platforms show a high degree of immune response and complement activation.

The hydrophilic hydrogel systems, especially the starch derivatives and dextrans have the advantage of being biodegradable, biocompatible and also largely transparent to the reticuloendothelial systems. They suffer the disadvantage of not being able to sustain

and control release of incorporated small molecules. When crosslinked in the bulk, they show more promise in sustaining the release of incorporated bioactive molecules, but bulk crosslinking raises the concern of denaturation and deactivation of delicate macromolecules like proteins. Additionally, this kind of bulk crosslinking may actually incorporate the bioactive molecule to the polymer thus making them unavailable for release.

Consequently the best approach seems to be to attempt to achieve the best properties of both types of systems. The inherent advantages of the hydrogel systems in terms of hydrophilicity and thereby lack of immune activation can be used as an advantage. An attempt to control the diffusional release of the incorporated bioactive molecule by formation of a semi permeable rate limiting membrane on the surface of the particle by a suitable interfacial crosslinking reaction may have significant potential. The advantage of an interfacial crosslinking reaction over a bulk crosslinking reaction is that the former is expected to leave the incorporated bioactive molecule unaffected within a benign hydrophilic core.

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III. HPLC ANALYSIS OF ALIPHATIC AND AROMATIC DICARBOXYLIC ACID CROSS-LINKERS HYDROLYZED FROM CARBOHYDRATE POLYESTERS FOR ESTIMATION OF THE MOLAR DEGREE OF SUBSTITUTION

ABSTRACT

Quantitative analysis of carboxylic acids in hydrophilic matrixes remains a challenge despite the introduction of derivatization reagents for carboxylic acids nearly three decades ago. Analysis of many bifunctional ester forming polymer cross-linkers are additionally complicated by their amphiphatic character. We report a sensitive and selective HPLC method to quantify organic dicarboxylic acids resulting from the hydrolytic degradation of cross-linked carbohydrate polyesters. An important group of cross-linkers encompassing C_7-C_{10} aromatic and nonchromophoric aliphatic diacid chlorides were investigated. Specific validation is reported for terephthalic acid, diethylmalonic acid and sebacic acid. Linear calibration curves were obtained in the 0.5 - 15.0 μg/mL range corresponding to a mass fraction of 0.05 - 1.5% diacid to polyester. The accuracy and precision in this range was 94.4 - 114.1% and 6.1 - 24.8% RSD, respectively. This method is suitable for the routine determination of the molar or mass degree of substitution in carbohydrate polyesters.

1. INTRODUCTION

Carbohydrates are the most abundant natural polymers available on earth. Among them, starch, in all its natural and semi-synthetic forms, is of great interest to food and pharmaceutical scientists because of its inherent advantages regarding safety and biocompatibility. Unmodified starch has limited application because of the undesirable properties of gelation and retrogradation. Cross-linking starch polymer molecules with bifunctional reagents by far the most important chemical modification used to control and minimize these undesirable properties (Langan, 1986), as well as to be used to create desirable properties such as in the development of biodegradable drug delivery devices. In this context, material development requires a routine method which is efficient and sensitive to quantify organic dicarboxylic acids resulting from modified carbohydrate polymers in order to relate the degree of cross-linking to physical, structural or chemical properties of the polymer.

Since most polyesterification reactions result in only a 0.5–2.0% degree of molar substitution of cross-linkers in the polymer (Jarowenko, 1986), sensitive methods of detection of the diacid cross-linkers are required. In addition, many dicarboxylic acids commonly employed as cross-linkers have limited solubility profiles in aqueous and organically based solvent systems. For instance, terephthalic acid, an important cross-linker in condensation polymerizations, is practically insoluble in any solvent other than aqueous alkali.

Although many methods have been reported for the analysis of monocarboxylic acids (Coenen, A. J. J. M., 1992; Toyo'oka, T., 1995; Mehta, A., 1998), only a few methods have been reported for the analysis of dicarboxylic acids (Fischer, K., 1997;

Baziramakenga, R., 2001; Grushka, E., 1975) with requisite sensitivities in the upper nanomolar to lower micromolar concentration range. Ion chromatographic methods (Fischer, K., Baziramakenga, R.) utilizing ion-exchange separation and conductance detection report the analysis of C_6 aliphatic dicarboxylic acids and a number of aromatic dicarboxylic acids with good sensitivity. The acidic mobile phase in one method (Fischer, K.), however, limits the method to only the most soluble of the aliphatic dicarboxylic acids. No accuracy and precision data are given for this method. The other method (Baziramakenga, R.), requires two separate chemical analyses using different analytical columns for the separation of aromatic and aliphatic dicarboxylic acids. Reported accuracy and precision for this method are good. Both methods require ion suppression to achieve optimal sensitivity. A gas chromatographic method using on-column trimethylsilane derivatization (Docherty, K. S.), is described for the analysis of C_2 - C_{10} dicarboxylic acids with excellent selectivity, but inadequate sensitivity.

Sensitive and selective HPLC methods for analysis of fatty carboxylic acids are reported based on derivatization with fluorescent chromogenic reagents (Naganuma H., 1989; Yamaguchi, M., 1990). These methods, however, require synthesis and purification of the fluorescent reagents which are not commercially available. Even with the power of tandem mass spectrometry, derivatization is necessary to obtain adequate selectivity in complex matrixes (Kushnir, M. M., 2001).

Reported reverse-phase HPLC methods (Grushka, E., 1975; Capristo, E., 1999) with potentially adequate sensitivity are based on the derivatization reaction of carboxylic acids with *p*-bromophenacyl bromide (Durst, H. D., 1975) or phenacyl bromide (Borch, R.F., 1975) both of which are commercially available. The utility of this reaction was

demonstrated for nonchromophoric C_4-C_6 dicarboxylic acid esters of phenacyl bromide, but not *p*-bromophenacyl bromide (Grushka,E.). These authors found the *p*-bromophenacyl derivatives of some diacids, especially fumaric and succinic, to be insoluble in almost all solvents. Phenacyl bromide derivatives showed improved solubility characteristics, but were still compromised. Chromatographic resolution of the dicarboxylic acid derivatives was poor and exhibited peak distortion on a nonyl bonded stationary phase column. Another study (Capristo, E.) showed much improved specificity and peak shape of detected dicarboxylic acids resulting from the *in vivo* deesterification of triglyceride esters of sebacic and dodecanedioic acids on an octadecyl bonded stationary phase. Analytical accuracy and precision was only provided with respect to the triglyceride esters for this method.

The purpose of this study was to develop a sensitive and selective HPLC assay for routine analysis of organic dicarboxylic acids resulting from the hydrolytic degradation of cross-linked polyester carbohydrates in order to determine the molar or mass degree of substitution from various polymeric materials. Particular emphasis was placed on assay methodology for an important category of dicarboxylic acid chloride cross-linkers encompassing C_7-C_{10} aliphatic and aromatic diacid chlorides. Validated analytical accuracy and precision is reported specifically for terephthalic acid, diethylmalonic acid and sebacic acid, although the method is suitable for a variety of C_6 diacids.

2. EXPERIMENTAL

2.1 Preparation of reagents and sources

Acetonitrile was used as a reverse phase HPLC solvent (Optima grade, Fisher Scientific/Acros Organics, Pittsburgh PA, USA). N, N-dimethylformamide (Fisher), glacial acetic acid (Fisher), ammonium hydroxide (Fisher) and potassium hydroxide (Fisher) were reagent grade. Terephthalic acid (Acros), phenacyl bromide (2-bromoacetophenone, Acros), sebacic acid (Sigma-Aldrich/Fluka, St. Louis MO, USA), diethylmalonic acid (Aldrich), suberic acid (Aldrich), azelaic acid (Fluka), and triethylamine (Sigma) were of purity >98%. Water for the mobile phase was double glass distilled. For the polymer condensation reactions, terephthaloyl chloride >99% (Aldrich), sebacoyl chloride 92% (Acros), and diethylmalonoyl dichloride 98% (Aldrich) were used as reagents.

Analytical derivatization standard solutions were prepared as follows. A 200 mM (40 mg/mL) phenacyl bromide solution was made by dissolving 400 mg phenacyl bromide in 10 mL of acetonitrile. A 60 mM triethylamine solution was prepared by adding 832 μL of triethylamine to a 100 mL volumetric flask and diluting to volume with acetonitrile.

2.2 Synthesis of carbohydrate polyesters

Polyesterification of a variety of starches and modified starches with different mass percentages of amylose and amylopectin were investigated. These included maltodextrin from corn starch (Maltrin M40, Grain Processing Corporation, Mucatine IA, USA), maltodextrin from waxy maize starch (C*Pharm 01980, Cerestar USA, Inc., Hammond IN, USA), corn starch (Sigma), high amylose corn starch (C*Amylogel 03003, Cerestar

USA, Inc.), waxy maize starch (PFP 2850, Cargill Foods, Minneapolis MN, USA), hydroxypropylated starch modified from tapioca starch (C*Aratex 7570, Cerestar USA, Inc.), and hydroxyethyl starch modified from corn starch (Sigma). Polyesterification of the starches was accomplished using an emulsion polymerization technique similar to that reported by Lévy and Andry (Lévy, M. C.,1992). Briefly, a specified carbohydrate was dispersed in aqueous 200 mM borate buffer, pH 10. Carbohydrate concentrations ranged from 3 to 20% (w/v) for starches and were 50% for maltodextrins. The dispersion was heated to approximately 100°C to produce a solubilized colloidal solution, and then allowed to cool to about room temperature. The aqueous colloid was emulsified with a continuum phase in a 1:5 solvent ratio, respectively, consisting of a mixture of cyclohexane and chloroform 68:32 (v/v) containing an appropriate surfactant in a homogenizer. After homogenization, an aliquot of cylcohexane/chloroform containing the diacid chloride is added to the emulsion to give a final concentration of 60 mM in the continuum phase. The reaction was carried out for 3 hours at ambient temperature with stirring. Esterified carbohydrate was precipitated by adding the reacted emulsion to excess ethanol while stirring. The precipitated residue was collected either by gravitational settling or centrifugation, decanting, and washed three additional times with ethanol. The product was dried in a vacuum oven at 20-25°C at 20 psi for subsequent analysis of cross-linking efficiency and as material to be used to determine the suitability of this assay.

2.3 Calibration standards, sample preparation, and polyester hydrolysis

Depending on the application, either terephthalic acid, diethylmalonic acid, or sebacic acid was chosen as the internal standard. The internal standard primary working solution was prepared by dissolving 40 mg of the appropriate acid in 100 mL of 5 M ammonium hydroxide to make a final concentration 400 μg/mL. The other two acids were regarded as analytes. Working solutions were prepared by dissolving 40 mg analyte with an appropriate volume of 5 M ammonium hydroxide to give two working solutions with final concentrations of 40 μg/mL and 400 μg/mL. Calibration standards were prepared by adding 500 μL of the 40 μg/mL analyte solution, and 100, 500, 1000 and 1500 μL of the 400 μg/mL analyte solution, respectively, to 4 mL screw-top glass vials containing 40 mg of nonesterified carbohydrate. A 1000 μL aliquot of the internal standard solution was added, and aliquots of 5 M ammonium hydroxide were added to each vial to give a total volume of 4.0 mL for each sample. For carbohydrate polyesters, 40 mg of the polymer, 1000 μL of the internal standard solution, and 3.0 mL of 5 M ammonium hydroxide were added to the vial.

The vials were tightly capped and hydrolyzed at 130°C in a dry bath for 60 min. After hydrolysis, the samples were allowed to cool to ambient temperature, and 1.0-mL aliquots of hydrolysate were transferred to 15-mL centrifuge tubes. A 9.0 mL aliquot of a 60 mM triethylamine in acetonitrile solution was then added to each tube in order to precipitate any remaining insoluble carbohydrate. The tubes were vortexed and centrifuged at 2,500 rpm for 30 min separating any insoluble material from the dissolved diacids. Exactly 1.0 mL of the clear supernatant was retained for subsequent analytical derivatization and transferred to a reaction vial. The theoretical final concentrations of

the calibration standards are 0.5, 1.0, 5.0, 10, and 15 μg/mL, corresponding to 0.5, 1.0, 5.0, 10, and 15 μg/mg carbohydrate, or 0.05 to 1.5 % (w/w) respectively.

2.4 Analytical derivatization

The 1-mL aliquot of retained supernatant from each sample is evaporated until completely dry in a vacuum manifold. It is important that sample is dry and any excess ammonium hydroxide is removed by evaporation of the ammonia. To each sample is added 300 μL dimethylformamide, 100 μL acetonitrile, 300 μL 60 mM triethylamine in acetonitrile, and 300 μL phenacyl bromide solution. The reaction vials are capped and heated at 60°C for 60 min in a dry bath. Following the reaction, the samples are removed from the heating block and allowed to cool to ambient temperature before subsequent processing.

2.5 Separation of soluble polysaccharides

It is necessary to reduce the soluble polysaccharide background of the sample resulting from the hydrolytic decomposition of polyesters and oxidative decomposition of the carbohydrate backbone. Although residual polysaccharides do not interfere with the derivatization, they can effect the chromatography. This separation was achieved by solid phase extraction using PrepSep (Fisher) C_{18} SPE cartridges (500 mg sorbent/6 mL). The SPE cartridge is conditioned by passing 4 mL of distilled water through the cartridge. The derivatized sample (1 mL) is diluted with 2 mL of distilled water and drawn through the sorbent with a vacuum manifold, washed with 4 mL water, and eluted with 1.0 mL of acetonitrile into autosampler vials.

2.6 Samples for derivatization reaction kinetics

Standard solutions of diacids used in the evaluation of the derivatization reaction kinetics were prepared by dissolving the diacids in an appropriate volume of 60 mM triethylamine in acetonitrile. An aliquot of 1.0 mL was evaporated until dry, and derivatized according to the above procedure. The samples were reacted at 60°C for varying periods of time. At the completion of each reaction period, sample vials were placed in a dry ice/acetone slurry in order to quench the reaction. Samples were retained in this bath until injected on-column for chromatographic analysis without further cleanup.

2.7 Chromatographic conditions and instrumentation

The chromatographic system consisted of ThermoSeparation (San Jose CA, USA) Model P2000 pumps used in the isocratic mode, Model AS3000 autoinjector, and Model UV1000 variable-wavelength UV detector. Data collection and processing used Chromquest v3.0 software and a PC computer. The stationary phase was a 25 cm x 4.6 mm i.d., 5 μm, 150 Å pore size, C_8 bonded phase on silica chromatographic column (Beta Basic 8, ThermoHypersil-Keystone, Bellefonte PA, USA) and a 10 mm x 4.6 mm i.d., Beta Basic 8 precolumn. The mobile phase consisted of 60% acetonitrile, 40% 100 mM acetate buffer at pH 4.5 delivered at a flow rate of 1.0 mL/min. The column temperature was ambient. The detection wavelength for all derivatized analytes was 242 nm. The injection volume was 15 μL.

3. RESULTS AND DISCUSSION

3.1 Phenacyl ester formation

The reaction of carboxylic acids with phenacyl bromide typically require the production of a “naked” carboxylate anion utilizing either triethylamine (Borch, R. F.,1995) or crown ethers (Durst, H. D.,1975) as catalysts. The proper choice of base used to hydrolyze carbohydrate polyesters is essential. The high concentration of base required for the hydrolysis precludes the use of alkali metal bases which have the potential to overwhelm the catalysts competitively with metal cations, as well as provide a large excess of hydroxide ions which are good nucleophiles for the production of phenacyl alcohols. Both of these reactions hinder the desired formation of phenacyl esters of the analytes. The use of ammonium hydroxide for hydrolysis was found to overcome these deficiencies since excess reagent can be evaporated leaving a stoichiometric 1:1 ammonium salt with carboxylate anion. The ammonium cation is displaced with excess triethylamine as the catalyst in the reaction solvent.

Another problem is reaction solvent choice. Although the aprotic solvent acetonitrile is generally reported as the preferred reaction solvent for fatty acid derivatization, reaction products from some diacids are insoluble (Grushka, E., 1975). The phenacyl ester derivatives of the diacids investigated in this study were generally found to be solubility limited in neat acetonitrile. Co-solvency with 30% dimethylformamide improved solubility of diacids and products in the reaction medium.

Terephthalic, diethylmalonic and sebacic phenacyl ester formation was facile under the prescribed conditions. The reactions kinetics (Figure1) showed completion of derivative formation within 40 min for all three diacids. The kinetic profiles were

monotonically asymptotic. Chromatographic analyses showed that the retention time (t_R) of all corresponding products were independent of mobile phase pH in a range from 2.9 to 7.0. In addition, phenacyl ester derivative kinetics remained stable beyond 40 min for reactions carried out to 2 hr. Although theoretically a mixture of both monoester and diesters products can be formed, no biphasic kinetic profile of the reaction products was observed corresponding to monoester formation, typical of A $\rightarrow$ B $\rightarrow$ C kinetics with B corresponding to the monoester and C the diester. In view of the pH independence of the reaction products, these results indicate that the phenacyl derivatives were diesters.

3.2 Chromatography and selectivity

Typical chromatograms obtained from spiked standards prepared with Maltrin M40 as the background, a blank sample with Maltrin M40, and authentic Maltrin M40 polyester crosslinked with terephthaloyl chloride are illustrated in Fig. 2 and 3.

Esterification of carboxylic acids with phenacyl bromide, produce a large number of reaction by-products, having retention times of < 10 min in this assay. These peaks are present in chromatograms from neat samples without a carbohydrate background as well as those with carbohydrates. Similar results in other matrixes using this reaction have been reported (Capristo,E., 1999). In developing the chromatography, the intent was to adequately separate analytes of interest by increasing on-column retention relative to the reaction by-products in order to increase selectivity.

Terephthalic acid, diethylmalonic acid and sebacic acid derivative retention times were 12.3 ± 0.11 (n=25), 15.9 ± 0.14 (25), and 19.5 ± 0.20 (25) min, respectively. The blank control sample was clean with respect to the diethylmalonic acid and sebacic acid derivatives. Low level interference with a similar retention time as terephthalic acid was

detected in the blank which limited the lower level of quantitation for terephthalic acid to about 0.5 μg/mL. Qualitatively, chromatographic profiles from blank samples using other carbohydrates resulted in virtually identical chromatography.

The suitability of this assay for other dicarboxylic acids including suberic and azelaic acids is demonstrated in Fig. 4. For C_5 dicarboxylic acids including succinic acid, retention was inadequate (< 10 min) resulting in reaction by-product interference. Since the retention time increases with diacid hydrocarbon length, this assay would be considered to provide adequate selectivity for dicarboxylic acids of C_6 hydrocarbon length.

Minimizing soluble polysaccharides in the injected samples was essential. During the development of this method and prior to adding an SPE cleanup step, it was noticed that analyses of an autosampler tray of samples resulted in progressively longer retention times of analytes to values more than double their initial values. Although the Beta Basic 8 column is specified by the manufacturer to be endcapped with a 7% carbon load, significant on-column accumulation of background polysaccharides was found, presumably due to their interaction with the silica support. This accumulation resulted in significant changes in chromatography probably due to mixed mode retention of analytes. Retention of the background polysaccharides relative to the analytes on a C_{18} SPE cartridge, however, was found to be minimal, and analyte extraction was optimized to wash out soluble polysaccharides and elute the purified analytes. Polysaccharide on-column buildup was found to be reversible such that columns could be regenerated by flushing with 8.5 mM aqueous ammonium hydroxide for 30 min followed by distilled water. Routine column cleanup is recommended for assay ruggedness.

3.3 Linearity of calibration curves

Calibration curves were constructed by statistically analyzing 5 calibration levels with 5 replications within each level. All samples contained Maltrin M40 as the background carbohydrate. These curves were evaluated with regard to peak area ratio and peak height ratio of a designated analyte relative to the internal standard. The selected internal standard was added at a concentration level to attain a final concentration of 10 μg/mL after hydrolysis and carbohydrate precipitation. For terephthalic and diethylmalonic acid standard curves, sebacic acid was chosen as the internal standard. For sebacic acid standard curves, diethylmalonic acid was chosen as the internal standard. Least-squares regression analysis used a model with peak area ratio or peak height ratio as the dependent variable and concentration level as the independent variable. Linear calibration curves were established for all three diacids in the 0.5 to 15 μg/mL calibration range (Table 1). Extended calibration ranges from 0.2 to 40 μg/mL tended to deviate from linearity at the upper end, while the 0.2 μg/mL level was determined to be below the limit of quantitation.

3.4 Accuracy and Precision

Accuracy of analyte determinations ranged from 94.4 to 114.1% for all three diacids with the exception of the 0.5 μg/mL sebacic acid concentration level (Table 2). The precision of estimates ranged from 6.1 to 24.8 % RSD. UV response limited the lower limit of quantitation for sebacic acid to 1.0 μg/mL, while terephthalic and diethylmalonic acids were sufficiently sensitive to give lower limits of quantitation of 0.5 μg/mL. Although assay reproducibility was considered acceptable, improved precision could be

expected based on duplicate sample analysis. This is particularly relevant with regards to diethylmalonic acid.

The effect of different carbohydrate matrixes was evaluated with regards to accuracy based on calibration curves employing Maltrin M40 as the background carbohydrate matrix. Calibration standards containing 10 μg/mL terephthalic acid and maltodextrin from waxy maize starch, high amylose corn starch, waxy maize starch, hydroxypropylated tapioca starch, and hydroxyethylated corn starch were analyzed. The relative accuracies compared to the assayed 10 μg/mL terephthalic acid concentration level with Maltrin M40 were 93.6% for waxy maize starch, 95.0% for maltodextrin from waxy maize starch, 92.4% for high amylose corn starch, 101.1% for hydroxyethyl corn starch, and 105.9% for hydroxypropyl tapioca starch. All assayed values were within the 95% confidence interval for terephthalic acid assayed in a Maltrin M40 matrix. These results indicate that the assay is robust with regards to different carbohydrate matrixes and polyesters.

3.5 Carbohydrate Polyester Degree of Substitution

The cross-linking efficiency of various polyesterified starches was analyzed to relate molar degree of substitution to material properties required for nanoparticulate drug delivery systems, and to evaluate the application suitability of this method. In general, all carbohydrates tested reacted to form three-dimensional structured nanocapsules (Figure 5). The molar degree of substitution per anhydroglucose unit ranged from 0.044 to 8.32% (Table 3).

Several structural variables can be identified from this preliminary study, which contribute to the efficiency of polyesterification and formation of nanocapsules. Based on the molar degree of substitution per anhydroglucose unit, higher molecular weight starches are 2.5 - 14.3 fold more reactive when compared to maltodextrins when reacted at equivalent mass concentrations of carbohydrate and using the same cross-linker. This increased reactivity suggests that the tertiary structure of high molecular weight polysaccharides contribute to either the accessibility or reactivity of glucose alcoholic groups. The more sterically hindered diethylmalonoyl dichloride was 10-fold less reactive than terephthaloyl chloride as a cross-linker.

Of the higher molecular weight starches, no differentiation in gross physical attributes was observed between the hydroxyethyl and hydroxypropyl substituted corn starches and unmodified corn starch.

4. CONCLUSION

It can be concluded that a sensitive and selective HPLC method has been developed suitable for the analysis of C_6 dicarboxylic acids hydrolyzed from carbohydrate polyesters. Both aromatic and nonchromophoric diacids can be analyzed using a single assay. This assay can be used for investigation of detailed structural assessment of carbohydrate polyesterifcation requirements for producing nanoparticulate drug delivery systems.

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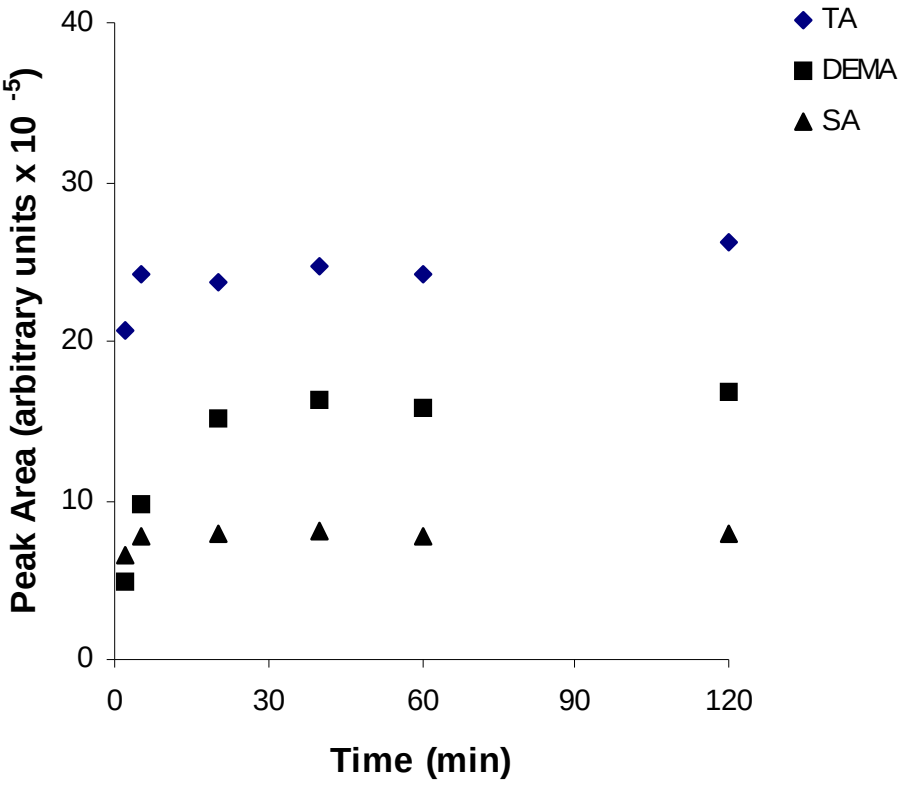


Figure 1. Phenacyl ester formation kinetics for terephthalic acid (TA), diethylmalonic acid (DEMA), and sebacic acid (SA) at 60°C.

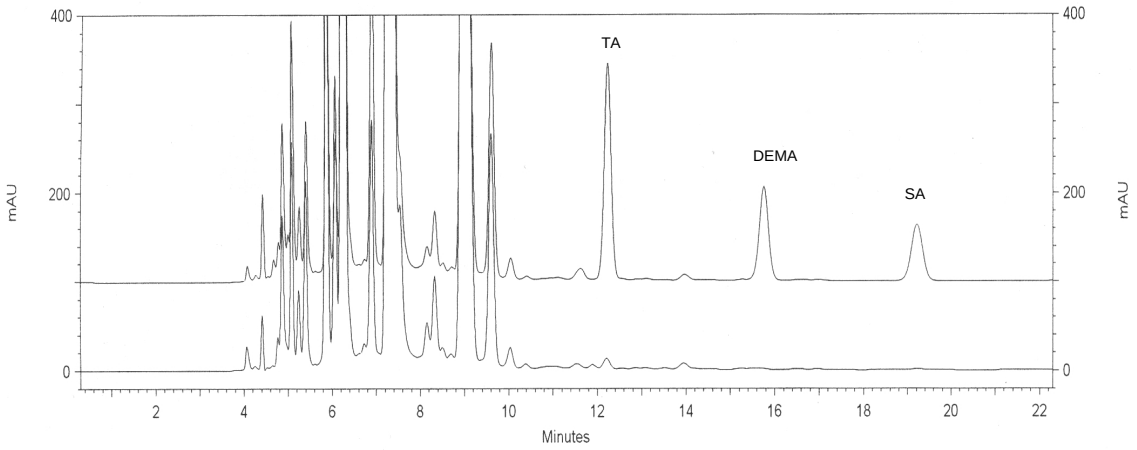
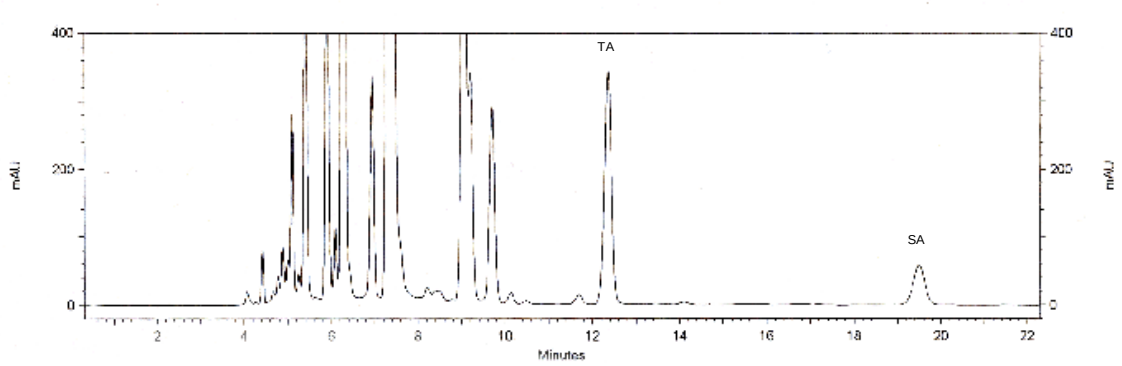
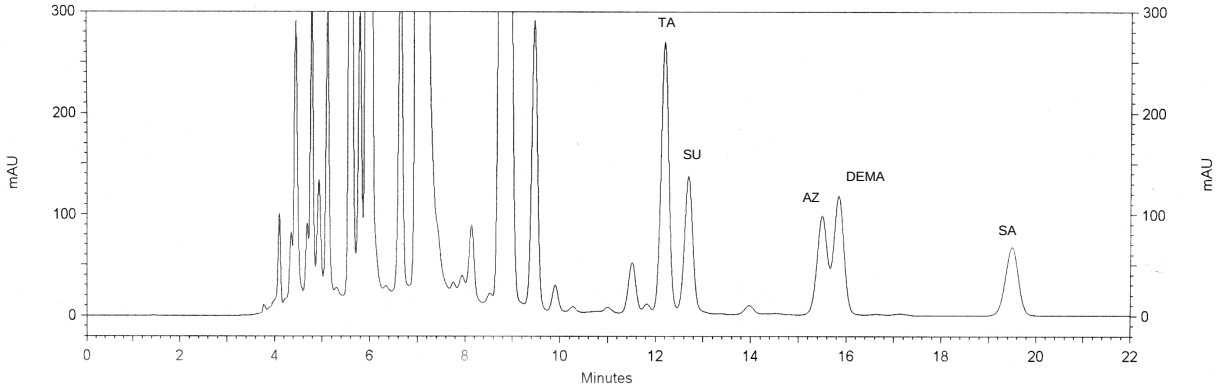


Figure 2. Chromatogram of dicarboxylic acid ester derivatives from a sample spiked with terephthalic acid (TA), diethylmalonic acid (DEMA), and sebacic acid (SA) corresponding to 10 μg/mL of each in hydrolysate with a Maltrin M40 (maltodextrin from corn starch) matrix background *(upper trace)*; blank sample containing Maltrin M40 matrix background *(bottom trace).*



Figigure 3. Chromatogram of an authentic sample resulting from the hydrolytic degradation of a carbohydrate polyester cross-linked with terephthaloyl chloride; sebacic acid (SA) is the internal standard; terephthalic acid (TA) is the diacid hydrolysis product.



Figigure 4. Chromatogram of C_6 dicarboxylic acids demonstrating utility of method for other diacids; terephthalic acid (TA), suberic acid (SU), azelaic acid (AZ), diethylmalonic acid (DEMA), and sebacic acid (SA).

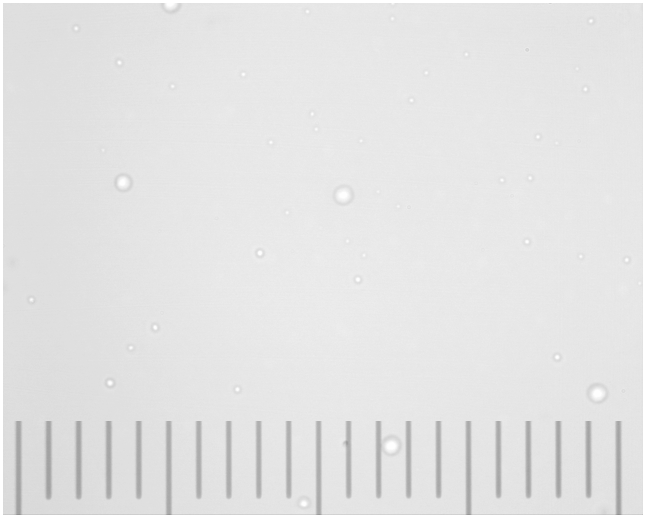


Figure 5. Optical micrograph of nanocapsules fabricated from the emulsion polyesterification of terephthaloyl chloride and corn starch. Nanocapsules are suspended in 100 mM phosphate buffered saline. Each division of the micrometer is 1 μm.

Table 1. Calibration curve statistics. [a] parametric standard error, [b] linear correlation coefficient.

Analyte	Peak Area Ratio			Peak Height Ratio		
	Slope (SE[a])	Intercept (SE)	r[b]	Slope (SE)	Intercept (SE)	r
terephthalic acid	0.2981 (0.00796)	0.151 (0.0667)	0.992	0.4848 (0.01131)	0.175 (0.0948)	0.994
diethylmalonic acid	0.2606 (0.01067)	-0.066 (0.0894)	0.981	0.3275 (0.01347)	-0.062 (0.1129)	0.981
sebacic acid	0.0325 (0.00099)	-0.00137 (0.008380)	0.989	0.0260 (0.00071)	-0.00209 (0.005965)	0.992

Table 2. Accuracy and precision for the determination of terephthalic, diethylmalonic, and sebacic acids hydrolyzed from carbohydrate polyesters. * N = 5, calculations based on peak height ratio from appropriate calibration curves. SD, standard deviation; RSD, relative standard deviation.

Analyte	Calibration Level (µg/mL)	Assay Mean* (µg/mL)	SD	Accuracy %	RSD %
Terephthalic acid	0.5	0.57	0.123	114.1	21.5
	1.0	1.09	0.145	109.0	13.3
	5.0	4.87	0.370	97.3	7.6
	10.0	9.81	1.116	98.1	11.4
	15.0	15.16	0.926	101.1	6.1
Diethylmalonic acid	0.5	0.52	0.093	104.2	17.9
	1.0	1.03	0.154	103.1	14.9
	5.0	4.91	0.651	98.3	13.2
	10.0	10.02	1.268	100.2	12.7
	15.0	15.01	2.314	100.1	15.4
Sebacic acid	0.5	0.65	0.225	130.5	34.5
	1.0	1.09	0.271	109.3	24.8
	5.0	4.93	0.737	98.6	15.0
	10.0	9.44	0.900	94.4	9.5
	15.0	15.39	1.086	102.6	7.1

Table 3. Estimation of bifunctional acid cross-linking efficiency in various carbohydrate polyesters.

Carbohydrate	Diacid Halide Cross-linker	Diacid Mass Fraction of Polyester (%)	Molar Degree of Substitution Per Anhydroglucose Unit (%)
maltodextrin from corn starch	terephthaloyl chloride	0.39	0.58
maltodextrin from corn starch	diethylmalonoyl chloride	0.041	0.044
maltodextrin from waxy maize starch	terephthaloyl chloride	0.57	0.84
waxy maize starch	terephthaloyl chloride	1.41	2.09
corn starch	terephthaloyl chloride	7.85	8.32
high amylose corn starch	terephthaloyl chloride	2.49	3.69
hydroxyethyl corn starch	terephthaloyl chloride	1.73	2.56
hydroxypropyl tapioca starch	terephthaloyl chloride	1.40	2.08

IV. SYNTHESIS OF CARBOHYDRATE NANOPARTICLES WITH HYDROPHILIC CORE

ABSTRACT

A new novel particulate drug delivery platform has been developed based on a fundamentally new approach. Existing particulate drug delivery platforms are all composed of hydrophobic polymers requiring the use of organic solvents in the internal dispersed phase through emulsion-solvent extraction/evaporation techniques. These solvents are frequently found to be incompatible with bioactive molecules especially macromolecules. Additionally, the surface of these hydrophobic polymers give rise to particle aggregation as well as complement activation resulting in fast removal of particulates from the body by the macrophages of the reticuloendothelial system. Thus systemic use of these platforms for sustained release and/or cellular level intervention is not possible. Other platforms using hydrophilic polymers like gelatin and also carbohydrate polymers have been explored. In these platforms the mechanical strength of hydrogels have to be reinforced by crosslinking the polymers throughout the device to prevent its disintegration upon exposure to aqueous based solvents. The crosslinking is generally performed with very reactive crosslinkers (Yong, S., 2005; Stenekes, R. J. H., 2000; Can, H. K., 2005) which can freely react with the bioactive molecule incorporated.

The novelty of this developed platform is that it is produced using a water-in-oil emulsion and interfacial crosslinking reaction (emulsion polymerization). The internal

aqueous phase containing carbohydrate polymer represents a benign and stabilizing microenvironment even for the most labile of bioactive molecules. Upon removal of water, the alcoholic hydroxyl groups of the carbohydrate polymer are expected to provide the hydrogen bonding required for the preservation of native biological activity of many macromolecules including proteins. Problems associated with protein denaturation are expected to be at least minimized both during synthesis and in the condensed solid phase. Since the polymers used in this platform are hydrophilic, complement activation and fast removal of the drug delivery system from the body is not anticipated. Additionally, the crosslinkers for interfacial crosslinking are chosen such that they are inactivated by reaction with water present in the dispersed phase. As a result, the crosslinking reaction involved in the preparation of the platform, is expected to be a purely interfacial phenomenon, keeping the core of the particles undisturbed and unaffected by the crosslinking. In the course of this investigation, preparation methodology of the nanoparticulate platform has been developed. Physical characterization has been carried out using both high resolution optical microscopy and transmission electron microscopy. Variables affecting the preparation of the particles have been identified and their contribution evaluated. Factors involved with incorporation of model molecules and their release from the platforms will be reported separately.

1. INTRODUCTION

The idea of using polymeric micro or nanoparticles as drug delivery platforms is reasonably well established. Delivery of drugs using these platforms have many advantages including improved bioavailability, stability, control over plasma

concentrations, duration of treatment and patient compliance, altering biodistribution (Couvreur, P., 1980) and above all maximizing the effectiveness of the drug at the site of action and minimizing the systemic side effects. Drug delivery research has been intensely focused on particulate platforms for more than three and a half decades since first observations with regards to attaining sustained release of drugs from polymer matrices (Yolles, S., 1971) were reported. Significant progress has been achieved in this field, from conceptualization to commercialization of some products delivering small molecules and polypeptides in a sustained manner from hydrophobic poly(lactide-co-glycolide) (PLGA) matrices (Tice, T., 2004).

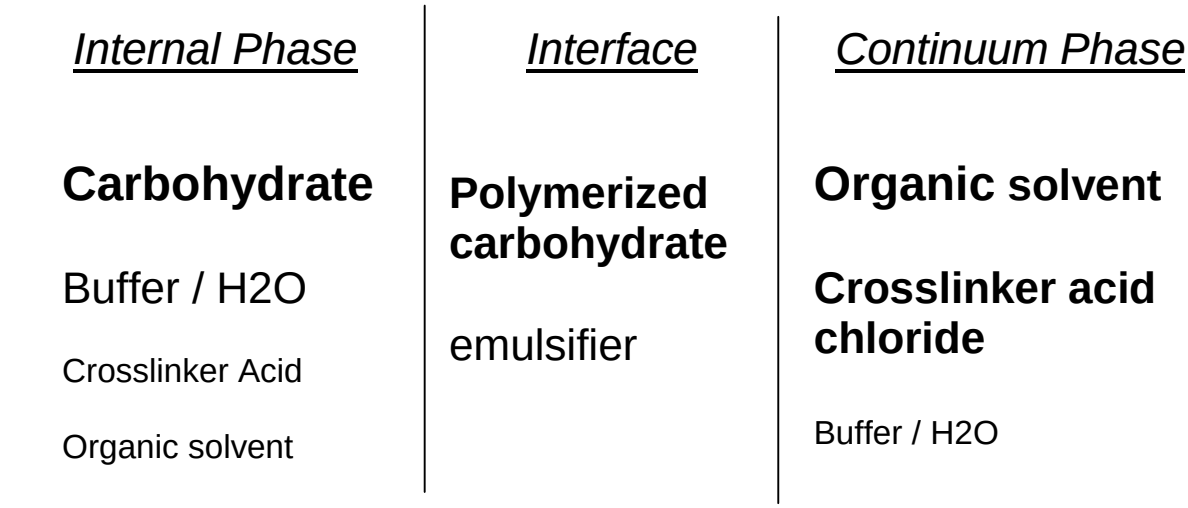
But, with current advances in molecular biology, genetics and biotechnology, the focus in drug delivery research is slowly shifting from mere sustained release of molecules into the blood stream to cellular level intervention in disease conditions. This kind of intervention using particulate platforms essentially requires the platform to remain in the systemic circulation for a long period of time. Limitations of traditional particulate platforms made from hydrophobic polymers in terms of aggregation and complement activation and their subsequent removal from the blood stream (Verrecchia, T., 1995) have excluded their use as systemically circulating delivery devices. They are, therefore not suitable for cellular level intervention which requires transport of the devices from the blood stream into target tissues. Surface PEGylation of nanoparticles prepared with hydrophobic polymers has been studied (Gbadamosi, J.K., 2002). It has been found that surface PEGylation frequently produces a heterogeneous population of particles with differential surface characteristics which is reflected in their physiological performance. When the heterogeneous populations of particles are sorted according to

their surface hydrophobicity by using hydrophobic interaction chromatography, a remarkable linear correlation between the particle zeta potential and sequestration by phagocytotic macrophage-type cells is found. The development of so called 'stealth' particles by covering hydrophobic surfaces with hydrophilic polyethylene polymers has only decreased the magnitude of the problem, but has not eliminated it.

Hydrogel systems composed of hydrophilic polymers have relatively greater biocompatibility. Complement activation is very low to negligible. Some hydrogels have been used as plasma expanders demonstrating their safety. On being introduced into water, hydrogel systems swell rapidly losing their mechanical strength and integrity with concomitant rapid release of the encapsulated biomolecules (Gombotz, W. R., 1995). In order to control the swelling, attempts have been made to reinforce the hydrogel structure by chemical crosslinking in the bulk. Polyfunctional aliphatic acids (Sediel, C., 2001), epichlorohydrin, phosphorous oxychloride, methylene-bisacrylamide (Denizli, B.K., 2004), and gluteraldehyde (Tabata, Y., 1989) have all been employed to modify hydrogels to improve the mechanical properties and decrease swelling which results in quick release of the target molecules from hydrogels. But crosslinking of hydrogels in the bulk, especially when they contain labile bioactive compounds like proteins, poses a significant problem of denaturation, aggregation or immobilization as a result of the crosslinkers reacting with the incorporated bioactive molecule.

In the context of current approaches and their limitations, a new and novel approach has been conceived as the operating principle of this investigation. The idea is to incorporate the bioactive molecules in the core of hydrogel nanoparticles fabricated through interfacial crosslinking carried out in a water-in-oil emulsion. If the crosslinkers

are carefully chosen in such a way that they are soluble in the continuous organic phase of the emulsion and are deactivated in the dispersed aqueous phase, the crosslinking reaction can only occur at the interface where the hydrophilic polymer and the crosslinker comes in contact.



Scheme 1. An illustration of concentration gradients of different component molecules in the emulsion polymerization system across the Gibb's thermodynamic interface between aqueous and organic phases. Bold type represents high concentration and small type represents low concentration.

The emulsion interfacial crosslinking/polymerization conception can be better understood with an illustration of the principle of Gibb's thermodynamic interface (Scheme-1). As is evident in the diagram, the carbohydrate polymer and buffer concentrations are high in the aqueous internal phase and very low in the organic phase. The emulsifier, by its nature is mostly concentrated at the interface. Crosslinker acid chlorides are in high concentration in the continuum phase and low in the dispersed aqueous phase. Moreover, acid chlorides present in the aqueous phase in low concentrations are hydrolyzed into the parent acids. The interface between the

continuum and dispersed phases is the only region where both the carbohydrate and acid chloride crosslinker come in contact with each other in appreciable concentrations. Thus the resulting crosslinking reaction predominates at the interface to produce a crosslinked membrane at the surface of the nanoparticle. The membrane, thus produced is expected to improve properties like strength, rigidity, and maintain the integrity of the nanoparticles as well as the incorporated bioactive molecules. Additionally, the crosslinked membrane is expected to decrease swelling and the rate of diffusion of the incorporated bioactive compound out of the particles when resuspended in aqueous biological fluids.

One class of crosslinking reagents having suitable properties is the di or poly carboxylic acid chlorides. These acid chlorides are soluble in organic solvents used as the continuum phase of the emulsion and decompose on exposure to water to the corresponding acid and hydrogen chloride. They can however, react to the ubiquitous hydroxyl groups of the carbohydrate at the interface of the emulsion droplets to produce ester crosslinks. In this work, various organic polycarboxylic acid chlorides have been investigated as potential crosslinkers. The degree of substitution achieved has been determined in each case using high pressure liquid chromatography (HPLC). Three crosslinkers, terephthaloyl chloride, trimesoyl chloride and diethylmalonyl chloride have been found as potentially ideal candidates for this purpose. Attempts have been made to characterize the reaction for optimal conditions in terms of pH of the aqueous phase, concentration of the crosslinker in the organic continuum phase, concentration of the polymer in the dispersed aqueous phase, reaction time and molar concentration of the buffers used.

The influence of different starches or modified starch polymers on the extent of crosslinking has also been investigated. Specifically, commercially available corn starch, amylopectin (waxy maize starch), hydroxyethyl starch and maltodextrins from corn and waxy maize starches have been investigated as polymers for the drug delivery system. An attempt was made to determine which of the two constituent polymers of starch, amylopectin or amylose was more suitable for the nanoparticle formation. Amylose was derivatized with dihydroxypropyl group to improve its solubility. The derivatized amylose, henceforth referred to as dihydroxypropyl amylose (DHPA), was then used as a polymer for nanoparticle synthesis. The dihydroxypropyl group was specifically chosen for derivatization due to their toxicological safety (Langan, R.E., 1986) and favorable properties.

2. EXPERIMENTAL

2.1 Materials

Corn starch and hydroxyethyl corn starch (Sigma, St. Louis, MO), high amylose corn starch (C*Amylogel 03003, Cerestar USA Inc., Hammond, IA), waxy maize starch (PFP 2850, Cargill Foods, Minneapolis, MN), hydroxypropylated starch modified from corn starch (C*Aratex 7570, Cerestar USA Inc., Hammond, IA) and maltodextrin (MA) from waxy maize starch (C*Dry 01955, Cerestar USA Inc.), maltodextrin (MCS) from corn starch (Maltrin M40, Grain Processing Corporation, Mucatine, IA) and amylose (City Chemicals, West Haven, CT) were used as supplied. Crosslinking acid chlorides include terephthaloyl chloride (TC) and diethylmalonyl dichloride (DEMC) (Sigma-Aldrich, St.-Louis, MO); trimesoyl chloride (TMC), sebacoyl chloride (SC) and oxaloyl chloride

(OC) (Acros Organics, Pittsburg, PA). Other reagents include glycidol, buffer salts (Sigma-Aldrich, St. Louis, MO); chloroform, cyclohexane, acetone, ethanol, triethylamine (TEA), phenacyl bromide (PB) and dimethyl formamide (DMF) (Fisher Scientific, Pittsburg, PA). All organic solvents were of ACS grade. Surfactants used were Tweens 40, 65, 80, and 85 (Fisher Scientific), nonyl phenyl ethoxylate (IgepalCO-520) and Spans 20, 40, 60, and 80 (Sigma-Aldrich, St. Louis, MO).

2.2 Synthesis of dihydroxypropyl amylose (DHPA)

Amylose derivatization was carried out according to a reported procedure (Wulff, G., 1998). Two (2) grams of amylose (average molecular weight 150,000 Daltons) was dispersed in 100 mL of 1(N) NaOH in a screw capped 1 L bottle after purging the alkali solution thoroughly with nitrogen and the cap tightly closed. The amylose is stirred on a magnetic stirrer for 2 hr or until the suspension becomes translucent. At this point, the bottle was opened, 6 ml of 2, 3-epoxy-1-propanol (glycidol, ~81 mMolar in reaction medium) was introduced and the bottle closed after thoroughly purging with nitrogen again. The reaction was allowed to continue overnight at room temperature. The reaction was stopped by neutralizing the base with HCl. The derivatized amylose was then precipitated by pouring the neutralized reaction mixture into 300 mL of ethanol. After 30 min, the precipitated derivatized amylose was filtered and redissolved in demineralized water. Alternate dissolution in demineralized water and precipitation in ethanol was repeated three times to eliminate the unreacted glycidol. Finally, the product was dried overnight in a vacuum oven at 54°C.

2.3 Emulsion stability

In order to obtain a stable emulsion, various water-in-oil systems were investigated. Cyclohexane, chloroform and cyclohexane/chloroform (68/32, v/v) mixed solvent systems were used as the continuum phase and aqueous buffers as the dispersed phase. Emulsion stability was investigated in pH 7.0 and 10.0 and a buffer strength of 200 mM. The volume ratio between continuum phase and dispersed was always maintained at 5:1. The surfactants Spans 40, 65, 80, 85 and nonyl phenyl ethoxylate were added in the continuum phase while the Tweens 20, 40, 60 and 80 were added in the dispersed phase because of their water solubility. Surfactants or surfactant combinations were evaluated in a concentration range of 1.0 to 6.0 % (w/v). The two phases, 20 mL aqueous and 100 mL organic, were mixed by homogenization (Silverson LR-4) at 5,000 rpm for one minute to make the emulsion and left standing at room temperature for stability studies. The stability was investigated visually every hour for 6 hr and thereafter at 12 hr and 24 hr. The most stable of emulsions was chosen for nanoparticle preparation.

2.4 Synthesis of carbohydrate nanoparticles

Carbohydrate polymers and oligomers were dispersed in appropriate buffer and heated in screw capped bottles to produce carbohydrate colloids for high molecular weight polymers and true solutions for maltodextrins. The concentration of the polymers varied from 4.5 to 50% (w/v) according to the specific polymers. An attempt was made in each case to achieve a near saturation concentration of each polymer when cooled to room temperature. The effect of pH on the synthetic reaction was evaluated by buffering the colloid/solution to appropriate pHs. Buffer strength was maintained at 200 mM

except where the effect of buffer strength on the degree of substitution was investigated. Phosphate, acetate and borate buffers were used for maintaining pHs ranging from 3-11. The colloid/solution was allowed to cool to room temperature while stirring before further use.

The continuum phase was composed of 68% cyclohexane and 32% chloroform (v/v). Six (6) percent (w/v) of the surfactant Span-40 was dissolved in the continuum phase to prepare an organic phase designated as OP-1, which was subsequently used for emulsification. Twenty ml of the aqueous phase was poured into 100 mL of OP-1 and homogenized in a Silverson-LR4 homogenizer with a rotor-stator arrangement at 5,000 rpm for 1 min.

Terephthaloyl chloride (TC), trimesoyl chloride (TMC), diethylmalonyl chloride (DEMC), sebacoyl chloride (SC) or oxaloyl chloride (OC) was added to the cyclohexane/chloroform (68/32, v/v) in appropriate concentrations. Thus, a final concentration of 55 mM was achieved to prepare the organic phase designated as OP-2, except where mentioned otherwise. The crosslinker concentration was varied from 0.8 to 161 mM in the continuum phase in batches made for evaluating the effect of crosslinker concentration on degree of substitution. Two hundred (200) mL of OP-2 was added to the prepared emulsion as described and the reaction was allowed to continue under gentle stirring using a magnetic stirrer for an appropriate time. The reaction was stopped by pouring the reaction mixture into 700 mL ethanol in a 1000 mL beaker while stirring. The stirring was discontinued after 30 min and the formed nanoparticles slowly precipitated. Next the supernatant was decanted, the precipitate sedimented for 2 hr, and the collected precipitate was washed three times with ethanol followed by three washes

with acetone by repeated centrifugation at 1,500 g and resuspension by vortexing. Finally, the suspension of nanoparticles in acetone was dried under vacuum at 14.5 psi pressure at room temperature overnight.

2.5 Physical characterization

2.5.1 Particle size

The particle size was determined using photon correlation spectroscopy with a Nicomp model 380 (Particle Sizing Systems, Santa Barbara, CA) particle size analyzer. Average particle sizes based on population distributions were determined using Nicomp CW380v1.51A software. Measurements were made with a very dilute nanoparticle suspension (~40 μg/ml) in deionized water. Mean diameters were calculated from the volume and number averaged size based on discrete mono disperse distributions. Particle size measurements corresponded to the swollen mean diameters of the nanoparticles.

2.5.2 Visualization

Visualization of the nanoparticles and particle morphology was accomplished using transmission electron microscopy (TEM) and optical microscopy. For TEM a very dilute suspension (~40 μg/ml) of nanoparticles was made in deionized water. A drop of the nanoparticle suspension was placed on a copper grid covered with a polyvinyl alcohol film. The solvent was evaporated in vacuum. A microphotograph was then taken with an electron microscope (Zeiss, Model EMCR-10, Germany).

Since the nanoparticles can be visualized by TEM in the dry state only, visualization in aqueous suspension was achieved using an optical microscope (Olympus, Model BX-

51, Japan) used in the bright field mode. A digital camera (Olympus, Model CMAD-3, Japan) was used to capture the image.

2.6 Chemical characterization

Nanoparticles were chemically characterized in terms of the crosslinker content per unit carbohydrate mass and the molar degree of substitution per 100 anhydroglucose units. The molar degree of substitution (DS) was determined using the following equation.

$$DS = \frac{(\%CL \,/\, Mol.Wt.CL) * 100}{(100 - \%CL) / 162.14}$$

Here DS indicates number of crosslinker molecules reacted to each 100 anhydroglucose units and % *CL* is the (w/w) % of the crosslinker determined in blank nanoparticles when dry. Crosslinker di- and tri- carboxylic acids were quantitatively analyzed by an HPLC method developed for this purpose (Basu Sarkar, A., 2005) after hydrolysis of the crosslinked polymers. Briefly, required quantities of nanoparticles were suspended in 5 M ammonium hydroxide solution in a screw capped vial and heated to 130°C in a dry bath for 1 hour to hydrolyze the ester bonds between the carbohydrate and the crosslinkers. The vials were then allowed to cool down to room temperature, 1 mL aliquot of the hydrolysate was transferred to a 15 mL glass test tube and a 9 mL aliquot of 60 mM TEA in acetonitrile was added. After mixing the contents, the carbohydrate was precipitated by centrifugation at 2500 g for 30 min. One (1.0) mL of clear supernatant was transferred into a screw capped vial. Solvent was evaporated and the dry samples

used for derivatization. Derivatization was carried out by adding 100 μL acetonitrile, 300 μL of 60 mM TEA solution in acetonitrile, 300 μL of 200 mM PB solution in acetonitrile and 300 μL DMF and then heating at 60°C for 1 hour. The derivatized acids were further purified in a PrepSep C_{18} (Fisher) solid phase separation cartridge (SPE) (500 mg adsorbent, 6 mL volume). The SPE cartridge was conditioned by passing 4 mL deionized water prior to use. The derivatized sample was diluted with 2 mL deionized water and then passed through the cartridge. The sample was washed with 4 mL of distilled water and finally, the sample was eluted with 1 mL of acetonitrile into an autosampler vial for HPLC analysis.

3. RESULTS

3.1 Physical characterization

3.1.1 Particle Size

Particle size analysis showed that carbohydrate nanoparticles synthesized by emulsion/polymerization were generally in the size range of 100 to 300 nm using the specified conditions. The nanoparticle suspension in deionized water exhibited a single peak (Figure 1). The abscissa in this figure is in log scale. A close correlation was observed among the number weighed, volume weighed and intensity weighed values indicating a mono disperse particle distribution of a single population of particles.

3.1.2 Particle Morphology

Both optical and transmission electron microscopy were employed to investigate nanoparticle morphology. Optical microphotographs (Figure 2a-2f) indicate that

nanoparticles can be synthesized with all the carbohydrate polymers investigated. Fully hydrated particles could be seen as distinct structured vesicles with spherical shape. When suspended in distilled water, the particles suspended nicely, showing a mononucleated suspension. This observation, coupled with the observation that when particles were put into a non polar organic phase like cyclohexane or chloroform, they show a highly aggregated dispersion (photograph not shown), indicates that the surface of the nanoparticles is hydrophilic in nature as expected. The nanoparticles remained stable in aqueous suspension for at least 4 weeks (Figure 2e and 2f). Nanoparticles prepared from DHPA were initially distinct structured vesicles when suspended in water but dissolved completely in two days indicating that the DHPA particles were too water soluble. Another important observation from optical microscopy was that in some cases the nanoparticles synthesized from TC batches showed dark crystal-like spots within the particles. Terephthalic acid is known to be very insoluble at neutral pH. It was thus postulated that some terephthalic acid, generated as a byproduct of the reaction with water, accumulated within the nanoparticles and was not extracted during the washing process with ethanol and/or acetone. This was confirmed by the observation that the dark spots disappeared on addition of alkali in the suspension media. Transmission electron microscopy (TEM) (Figure 3a and 3b) shows slightly distorted spheres which result from the contraction of carbohydrate polymers in dehydrated particles. These observations also indicate partially hollow sphere cores. Additionally, the size estimates from TEM closely matches with the size range obtained from photon correlation spectroscopy.

3.1.3 Emulsion stability

Stable emulsions were found using Span 40 and Span 60 at a 3.0 % (w/v) concentration in cyclohexane, chloroform and cyclohexane/chloroform (68/32, v/v) as the continuum phase. The cyclohexane/chloroform mixed solvent system was stable over days probably because of the density matching with the dispersed phase at the chosen proportions of cyclohexane and chloroform.

3.2 Chemical Characterization

3.2.1 Relationship between polymer species and degree of substitution (DS) of TC

Synthesized nanoparticles, using DHPA, amylomaize starch, corn starch, amylopectin, HES, HPS, MCS and MA as condensing polymers, were evaluated. Each polymer or oligomer was reacted with TC for 3 hr at pH 10.0. The concentration of polymers in the aqueous phase was kept near saturation (Table 1). Initially, pH 10.0 was chosen for poly esterification since other workers found pH 9.8 to be optimal for similar reactions (Levy, M. C., 1991).

Results show that nanoparticles can be prepared with all the carbohydrate polymers and oligomers including MCS and MA. It was found that the lower molecular weight carbohydrate polymers, like MCS and MA (~3,200 Daltons), resulted in lower degrees of substitution with values of 0.58 and 0.84 per 100 anhydroglucose units. Higher molecular weight polymers all showed higher DS with values ranging from 2.08 for HPS to 8.32 for corn starch. Generally, amylose rich starches showed higher DS. The DS of TC with DHPA was 5.57, followed by amylomaize starch (~70% amylose) with a value

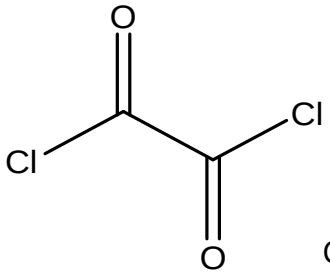
of 3.69, and waxy maize starch (no amylose) with a value of 2.09. The primary alcoholic groups of HES and HPS did not show any higher reactivity in terms of DS contrary to expectations. The very high reactivity of corn starch (DS of 8.32) could not be explained.

3.2.2 Effect of crosslinker species on DS

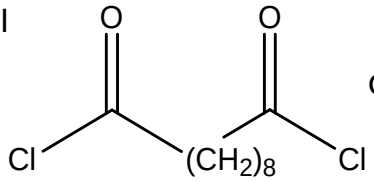
Five different di or tri functional acid chlorides, both aliphatic and aromatic, have been investigated for their suitability for this emulsion- interfacial crosslinking reaction. The acid chlorides include OC, SC, DEMC, TC and TMC. For comparison of reactivity all crosslinkers were evaluated at a 55 mM concentration in the organic continuous phase. When bifunctional linear aliphatic crosslinkers including OC and SC were used, nanoparticles could not be prepared since the emulsion broke and the phases separated. These results suggest that the surfactant sorbitan monopalmitate, which has alcoholic hydroxyl groups, react with the acid chlorides. Esterified surfactant thereby loses its amphipathic character bringing about phase separation. When sterically hindered aromatic di and tri functional acid chloride were used, emulsions were stable and nanoparticles could be formed.

Structural comparison shows that both the TC and TMC have a bulky benzene ring in between the reactive functional groups. From these observations it was hypothized that the bulky benzene ring produces enough steric hindrance with surfactant molecules to prevent them from reacting and thus prevent phase separation. In order to validate this hypothesis, DEMC, an aliphatic acid chloride crosslinker with two ethyl side chain was selected for evaluation. When DEMC was used as the crosslinker, indeed, no phase separation was detected. Thus, it could be established that one of the structural

requirements for the di or tri functional crosslinker to work in this system is that a bulky group/side chain has to be present in between the acid chloride groups of the crosslinkers to produce enough steric hindrance and prevent the crosslinker from reacting with the surfactant.



OC



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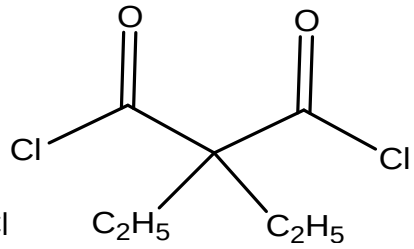
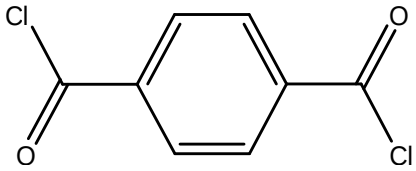
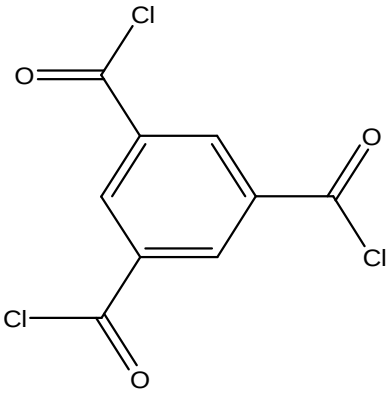


DEMC



TC



TMC

In order to assess the relative reactivity of the different crosslinkers, nanoparticle batches were made using MCS and DHPA as polymers and TC, TMC and DEMC as crosslinkers at pHs of 7.0 and 10.0. The crosslinker concentration in each case was fixed to 55 mM in the external organic phase. The reaction time in each case was fixed for 3 hr except for batches at pH 10.0 using DHPA as the polymer, in which case, the batches were reacted for 24 hours.

DEMC was found to be the least reactive crosslinker (Table-2). At pH 7.0, its reactivity ranged from 20-fold to 17-fold less for MCS and DHPA, respectively, than TC with the same polymers. At pH 10.0, the difference was less pronounced, such that the reactivity of DEMC ranged from about 10-fold and 5-fold less for MCS and DHPA, respectively, than TC. The number of crosslinker molecules reacted to 100 anhydroglucose units was relatively higher for batches with DHPA at pH 10.0 because those batches were reacted for 24 hr instead of 3 hr as in the case of other condensing reactions.

TMC was found to be the most reactive crosslinker. At pH 7.0, the reactivity of TMC was 1.54 to 1.38 times of that of TC with MCS and DHPA, respectively. The same trend in reactivity was also observed at pH 10.0. At pH 10.0, the reactivity of TMC was about 1.6 times greater that of TC with MCS and DHPA, respectively. The reactivity difference between the polymers DHPA and MCS were not pronounced at short reaction times.

3.2.3 Effect of pH on the degree of substitution

The effect of pH on the degree of substitution was investigated over a wide range of pH from 3 to 11. To avoid polymer specific trends, two different polymers, MCS and DHPA, were used for this purpose.

Esterification with TC (Figure 4 & 5) was found to be independent of pH between values of 7 and 10 exhibiting evidence of base catalysis above pH 10 and acid catalysis below pH 7. Esterification with TMC (Figure 6) was also generally independent of pH between pH values of 3 to about 7. Thereafter base catalysis contributed to increasing

DS. Esterification with DEMC (Figure 7) was independent of pH from values of 3 to 9 with base catalysis indicated at pH 10. A precipitous decline in DS was observed at pH 11 for TMC condensed with MCS, and DEMC condensed with DHPA. These results indicate that ester formation is overwhelmed by ester hydrolysis at pH ≥ 11. Similar results are reported elsewhere (Levy, M. C., 1991), indicating that the optimal pH for nanoparticle synthesis is pH 9.8. This investigation though, shows a much broader range of pH for optimal nanoparticle formation. Both acid and base catalysis, as observed in course of this investigation, are typical of ester formation reactions.

3.2.4 Effect of buffer strength on degree of substitution

Two series of nanoparticle batches were made with TC and TMC (Figure 8) with varying buffer strength in the dispersed aqueous phase. The TMC batches were made at pH 7.0 while the TC batches were made at pH 10.0. It was found that TMC reactivity was independent of the buffer strength over a wide range of concentrations. The reactivity of TC was found to be somewhat dependent on buffer strength at the low end. The reactivity of TC was found to be 5-fold less at buffer strength of 50 mM than 100 mM. In the range of 100 to 200 mM the reactivity of TC remained essentially the same.

3.2.5 Effect of polymer and crosslinker concentrations on the degree of substitution

Nanoparticle batches were made with TMC and DHPA at pH 7.0 and three different polymer concentrations of 1.5, 3.0 and 4.5 % (w/v of the aqueous phase). Results (Figure 9) show that the number of crosslinker molecules reacted to 100 anhydroglucose units

increases from 2 for a polymer concentration of 1.5% (w/v) to 2.9 for a polymer concentration of 3% (w/v) and finally to 4.6 for a polymer concentration of 4.5%. A concentration of 4.5% (w/v) is the maximum amount of the dihydroxypropyl derivatized amylose that can be dissolved in water.

Results from experiments carried out to establish the effect of crosslinker concentration on the degree of substitution were similar. Nanoparticle batches were made with MA as the polymer and TMC as the crosslinker under identical conditions. The only variable was TMC concentration which was varied from 0.8 to 162 mM. Results (Figure 10) show that the number of crosslinker molecules reacted to each 100 anhydroglucose units increased moderately from 0.03 for 0.8 mMolar TMC concentration to 1.07 for 80.86 mM TMC concentration and finally to 1.55 for 162 mM TMC concentration (Figure 10). The relationship between DS and crosslinker concentration was hyperbolic in the concentration range of 0.8 to 162 mM. These results indicate that carbohydrate polyesterification is dependent on both carbohydrate and crosslinker concentrations and suggest that the chemical kinetics is second order. Furthermore, in agreement with Le Chatelier's principle, the extent of reaction can be forced by increasing both crosslinker and carbohydrate concentrations. In view of the limited solubility of the higher molecular weight polymers, maltodextrins are therefore considered the preferred form of the carbohydrate reactant species for maximal condensation of the carbohydrate polyester. Nanoparticles formed with condensed maltodextrins will also contain the highest degree of carbohydrate at the core and thereby maximal solid state stability of the encapsulated macromolecules. In addition, product variability can be minimized by standardizing the reaction with a saturated solution of

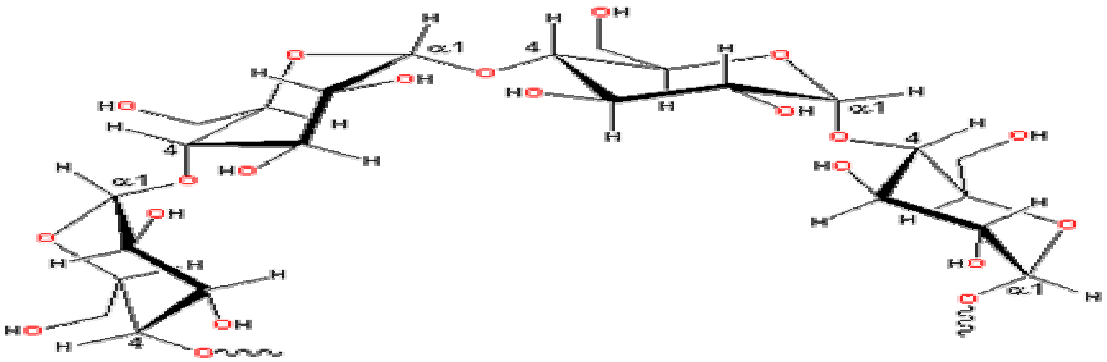
maltodextrin in the dispersed phase and varying the crosslinker concentration to obtain the desired performance characteristics.

3.2.6 Reaction time profile

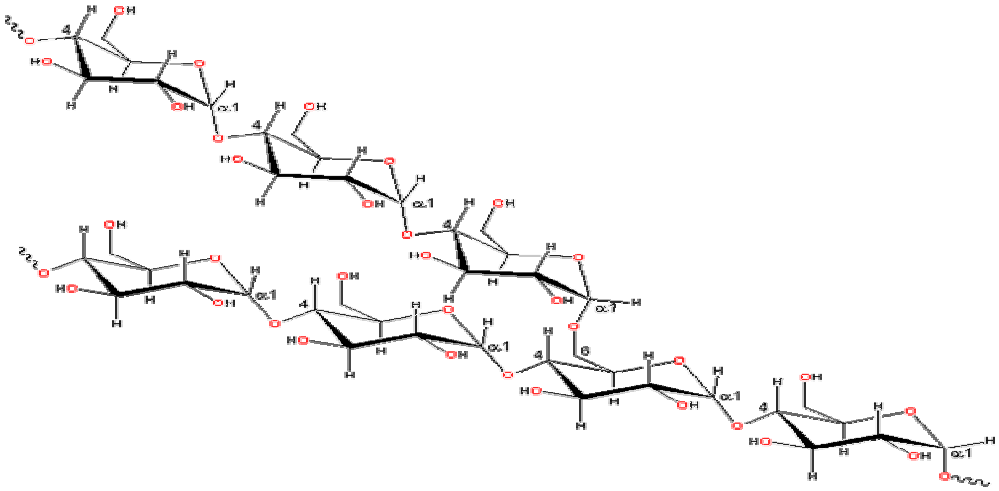
The time frame for the completion of the reaction was established using DEMC, TC and TMC crosslinkers and MCS as the polymer. Reactions were carried out under identical conditions with a 55 mM crosslinker concentration and 50% (w/v) polymer concentration. The only variable across the batches was reaction time for each series. The reaction time was varied from 1 to 48 hr for each series. The reaction was found to be complete in 12 hr for DEMC batches (Figure 11). For the TC and TMC batches, however the reaction was found to be complete in 24 hr (Figure 10).

4. DISCUSSION

Starch is a physical mixture of two different polymers; amylose and amylopectin. Amylose (Scheme 2) is a linear polymer composed of (1-4)- α-D glucopyranosyl units while amylopectin is a branched polymer consisting of both (1-4)- α-D glucopyranosyl units with occasional branching with (1-6)- α-D glucopyranosyl units. Because of the presence of a large number of alcoholic groups, starch polymers are hydrophilic (Domb, A., 1990). Additionally, the presence of a large number of reactive hydroxyl groups also presents an opportunity to crosslink starch through a multitude of different crosslinkers. The biodegradable nature of starches makes them one of the most suitable for particulate injectable DDS.



2 (a).



2 (b).

Scheme 2. Representative molecular structure of amylose (a) and amylopectin (b).

Though very similar in their molecular structure, amylose and amylopectin have very different properties. Amylose is sparingly soluble in water and retrogrades with time. Synthesis of nanoparticles with amylose is therefore, problematic. The reactivity and DS of this polymer may vary with the state of retrogradation which cannot be controlled. Amylose also produces inclusion complexes with a wide variety of molecules (Hui, Y., 1991; Kubik, S., 1996). The formation of inclusion complexes is not important for the synthesis of nanoparticles by itself, but it may have an important effect on encapsulation efficiency of different bioactive molecules.

Amylopectin on the other hand has relatively greater solubility and a relatively low grade of retrogradation. These properties make amylopectin a more suitable candidate for emulsion polymerization reaction. The fact that amylopectin is a branched polymer may also be helpful for the formation of a three dimensional crosslinked mesh membrane at the interface. A lower number of inter chain crosslinks should be required to achieve the same level of crosslinking density, as that with amylose. Crosslinking density is important for particle integrity in aqueous suspension and also for control of the release rate of incorporated bioactive molecules.

In addition to the physical integrity of the nanoparticles in physiological fluid, and the ability to incorporate and release bioactive molecules from the nanoparticles, several other physico-chemical properties are required for their use as circulating nanoparticles. For systemic use, the nanoparticles need to have the right surface polarity to be suspended in aqueous physiological fluid as discrete, mononucleated units. Otherwise they might aggregate to form bigger particles which might cause thrombosis in the smaller blood vessels. The fact that the nanoparticles synthesized in the course of this work, can be suspended as discrete, mononucleated particles is a major development toward their eventual use in the systemic circulation. The mono disperse nature of the produced nanoparticles is also helpful in controlling various important features including release rate.

The molecular weight of the starch polymers used for the synthesis of the nanoparticles is also important. The high molecular weight starches have low water solubility resulting in low polymer load of the dispersed aqueous phase. A low polymer load in the dispersed aqueous phase may affect the integrity of the nanoparticles and

more importantly, can potentially affect the encapsulation efficiency as a result of limited capacity of physical entrapment of the bioactive molecules by the polymer. High molecular weight starch polymers also show retrogradation. The effect of retrogradation of the starch polymers on their reactivity and DS has not been studied in the course of this work. But as the retrogradation process involves a change of the specific stereo chemical structure (Fechner, P.M., 2005) of the polymer, it is anticipated to have some effect on the reactivity and DS. In that case it will be impossible to precisely control the reaction with high molecular weight starch polymers if reacted during the retrogradation process. The low molecular weight maltodextrins, on the other hand, have two useful properties. They are much more soluble in water producing a clear true solution in as high as 50% (w/v) concentration and they do not retrograde. These two helpful properties outweigh their limitations regarding their relatively lower reactivity. Among the maltodextrins, MA is the more suitable polymer to be used for the synthesis of the nanoparticles as they are prepared from waxy maize starch (>99% amylopectin) and thus are branched to begin with. The problems associated with both polymer load and retrogradation can thus be addressed by using MA.

Another important feature of the nanoparticles to be used for systemic circulation is the size of the nanoparticles. As reported, nanoparticles could be prepared in the range of 100-300 nm (within colloidal size range). This size range is a very important property of the prepared particles for several reasons. Firstly, at this size range the particles have Brownian motion. This has been observed and recorded in a video. As a result of this Brownian motion the particles are not expected to sediment in circulating fluid. The phagocytotic uptake and removal of foreign particles are known to be size dependent

(Prabha, S., 2002) with minimum phagocytotic activity at the 100 nm size range. Thus the produced nanoparticles are expected to stay in the systemic circulation for a long period of time. Also nanoparticles in this colloidal size range can potentially escape the endothelial wall of the blood vessels especially in the areas of diseased patho-physiology. This particular feature of these nanoparticles exposes them to the possibility of cellular uptake through adhesion, cell fusion, passive endocytosis or active targeting.

The hydrophilic nature of the carbohydrate polymers are expected to be altered by the incorporation of the crosslinkers at the surface of the nanoparticles. In this regard, the two chosen crosslinkers have some theoretical difference. Terephthalic acid is a relatively non polar dicarboxylic acid with pKa_1 and pKa_2 of 3.51 and 4.82, respectively. But even at neutral pH, where both the acid groups are ionized, it is sparingly soluble in water. On the other hand, the more polar trimesic acid is a tri carboxylic acid with pKa_1 of 2.98 (other two pKa's are unknown), and is very soluble over a wide range of pH's. Thus, the nanoparticle batches produced using these two crosslinkers are expected to have a difference in polarity. A difference in polarity and wetting by water is indeed observed. Properly suspending the nanoparticles prepared with TC was more difficult; they tended to float on water due to lower polarity and higher surface tension. The TMC batches, on the other hand could be suspended readily. In the case of TMC, the probability of all three carboxylic acids crosslinking with carbohydrate is low. Thus the residual unreacted free carboxylic acids are believed to be partially responsible for making the surface hydrophilic reducing the interfacial tension in aqueous dispersed medium. The other favorable features of TMC are higher reactivity over a wide range of pH and near independence of reactivity on pH.

5. CONCLUSION

Several conclusions can be arrived at from these investigations. Firstly, carbohydrate nanoparticles can be made using the chosen emulsion-interfacial crosslinking method in the colloidal range required for their use in the systemic circulation. The hydrophilic carbohydrate polymers are ideally suited for this purpose. The nanoparticles produced have ideal surface properties of polarity for being suspended as discrete, mono dispersed, mono nucleated particles in aqueous media. Among all the carbohydrate polymers investigated maltodextrin from waxy maize starch has the most favorable properties for the preparation of the nanoparticles and their loading. Among the crosslinkers, only terephthaloyl chloride and trimesoyl chloride showed promise. Trimesoyl chloride is better suited because of higher reactivity, reactivity over a wide range of pH and lastly, but perhaps most importantly, because of the hydrophilicity it imparts to the surface of the nanoparticles.

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Table 1. Comparison of degree of substitution by terephthalic acid in preparation of nanoparticles with different carbohydrate polymers.

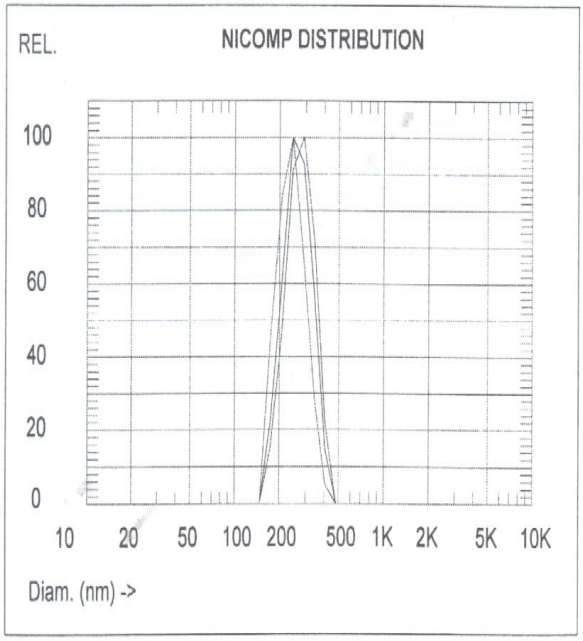
Polymer	Polymer Concentration (w/v)%	Degree of substitution *
Dihydroxypropyl amylose	4.5	5.57
Amylomaize starch	5	3.69
Corn starch	4	8.32
Amylopectin (Waxy maize starch)	3	2.09
Maltodextrin (from Corn starch)	50	0.58
Maltodextrin (from Waxy Maize starch)	45	0.84
Hydroxyethyl starch (from Corn starch)	20	2.56
Hydroxypropyl starch (from tapioka starch)	20	2.08

*Number of crosslinker molecules per 100 anhydroglucose units

Table 2. Comparative reactivity of the crosslinkers TMC, TC, DEMC at pHs 7.0 and 10.0 with polymers MCS and DHPA

Crosslinker	Number of crosslinker molecules reacted to 100 anhydroglucose units			
	POLYMER	MCS	POLYMER	DHPA
	pH7.00	pH10.00	pH7.00	pH10.00*
TMC	0.52	0.62	0.47	5.13
TC	0.34	0.39	0.34	3.13
DEMC	0.017	0.04	0.02	0.62

*Reacted for 24 Hours, TMC: trimesoyl chloride, TC: terephthaloyl Chloride, DEMC: diethyl malonyl chloride, MCS: maltodextrin from corn starch, DHPA: dihydroxypropyl amylose.



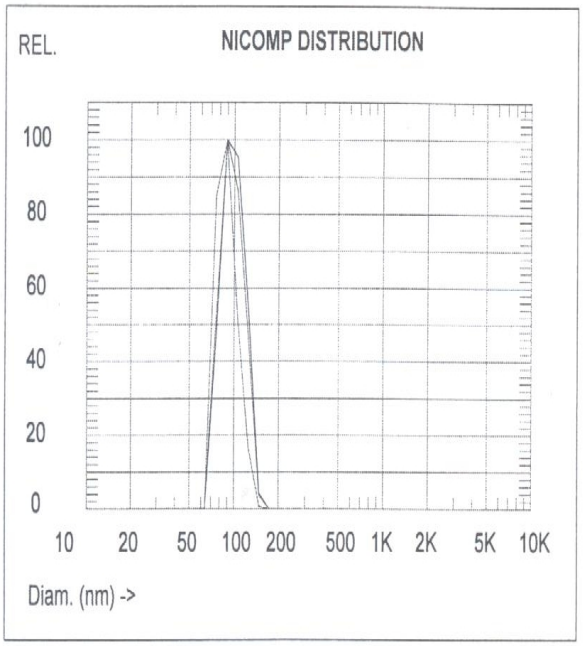


Figure 1. Particle size distribution of two representative nanoparticle batches. Nicomp® distribution is employed.

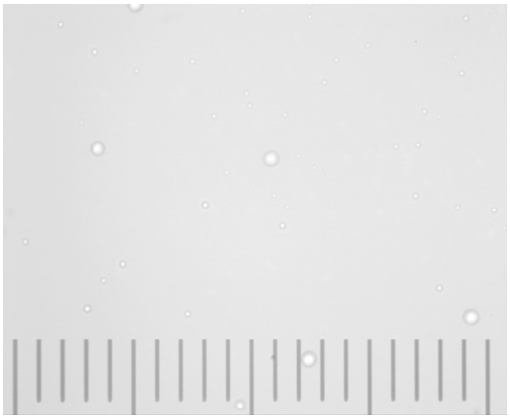


Figure 2a. Optical microphotograph of nanoparticles prepared from corn starch and terephthaloyl chloride and suspended in distilled water. Each division in the scale represents 1 μm. All other optical photographs are of same magnification.

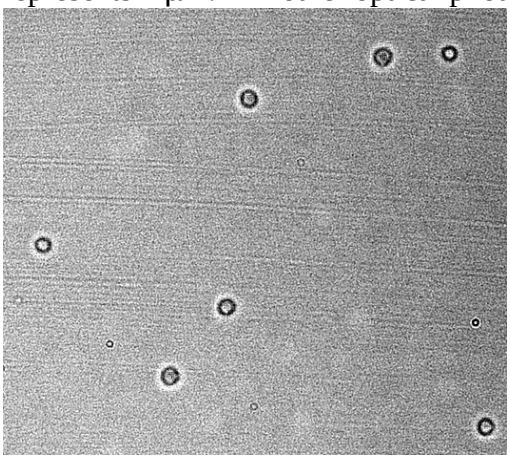


Figure 2b. Optical microphotograph of nanoparticles prepared from waxy maize starch and terephthaloyl chloride.

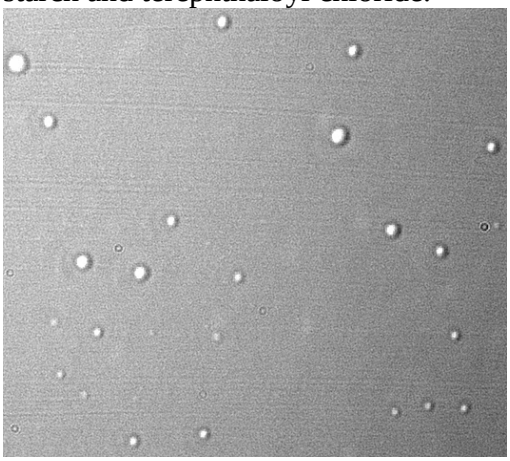


Figure 2c. Optical microphotograph of nanoparticles prepared from hydroxyethyl corn starch and terephthaloyl chloride.

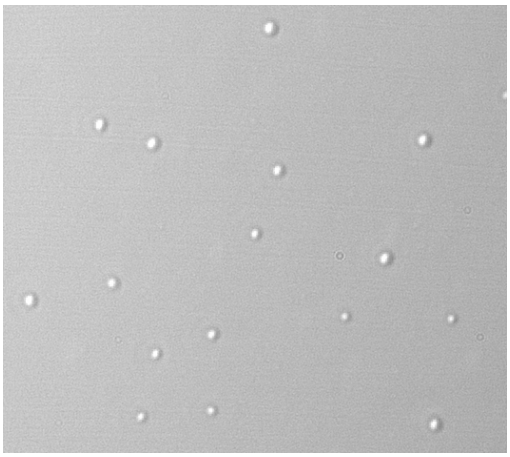


Figure 2d. Optical microphotograph of nanoparticles prepared from maltodextrin from corn starch and terephthaloyl chloride.

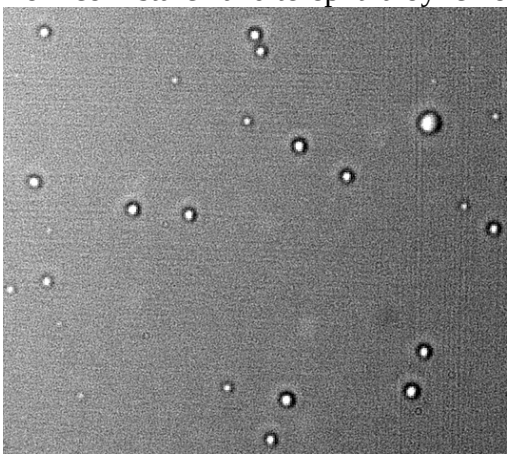


Figure 2e. Optical microphotograph of nanoparticles prepared from maltodextrin from waxy maize starch and trimesoyl chloride.

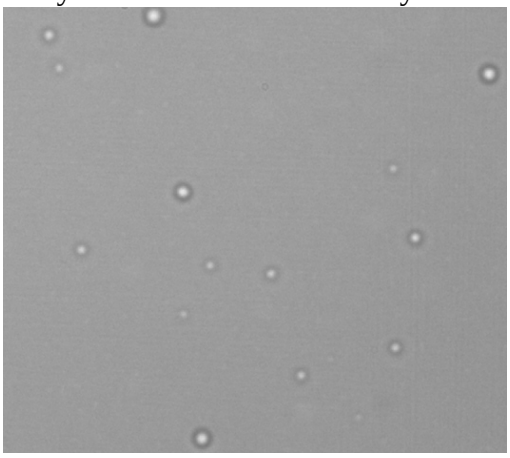


Figure 2f. Optical microphotograph of nanoparticles prepared from maltodextrin from waxy maize starch and trimesoyl chloride after 4 weeks in suspension in distilled water.

Figure 3a.

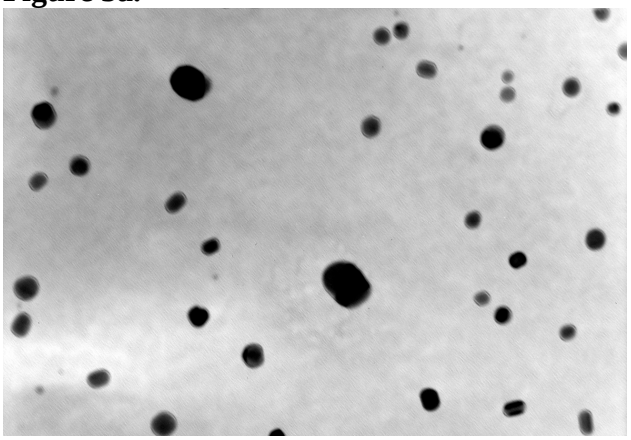


Figure 3b.

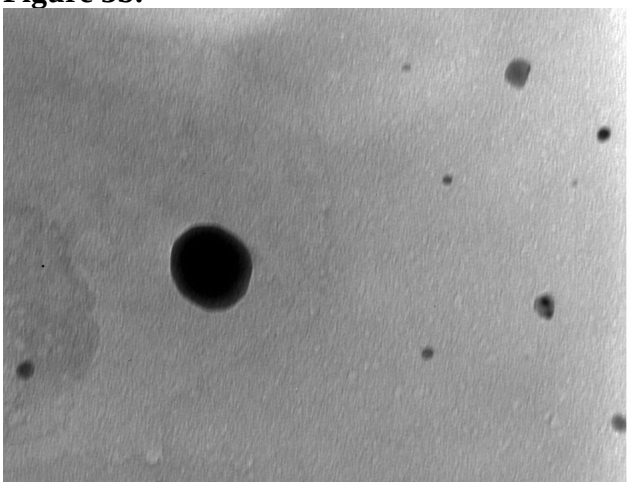


Figure 3. A representative transmission electron microphotograph of nanoparticles prepared with maltodextrin and trimesoyl chloride. The sample is dry-mounted on a copper grid coated with polyvinyl alcohol film. Magnification was (a) 100,000 (b)160,000.

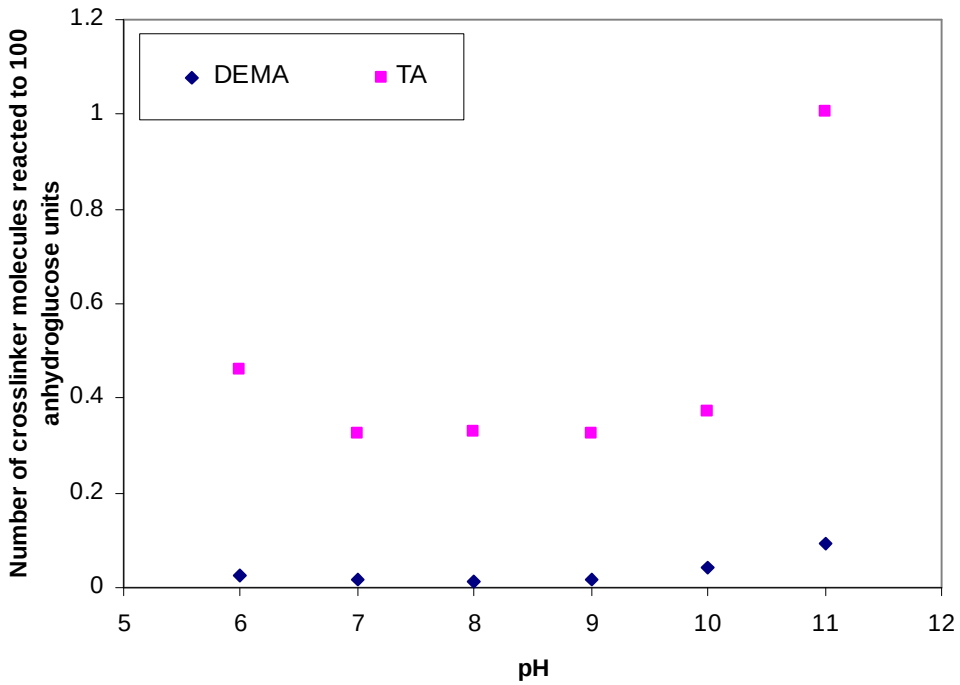


Figure 4. Effect of pH on degree of substitution with TC and DEMC as crosslinkers and maltodextrin (MCS). Reaction was carried out for 3 hr.

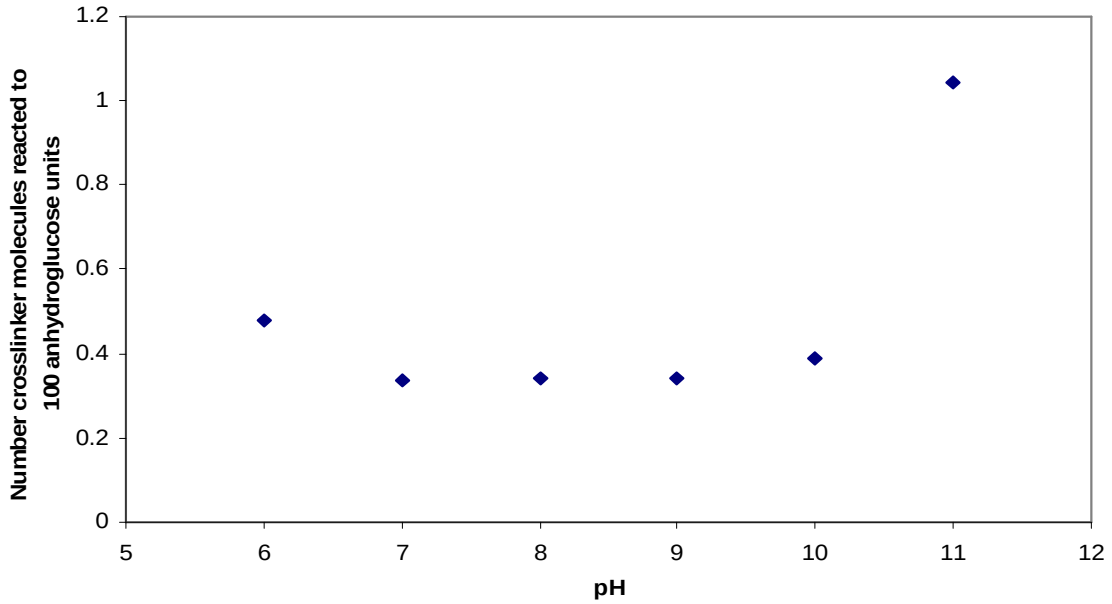


Figure-5. Effect of pH on degree of substitution with TC as crosslinker and DHPA. Reaction was carried out for 3 hr.

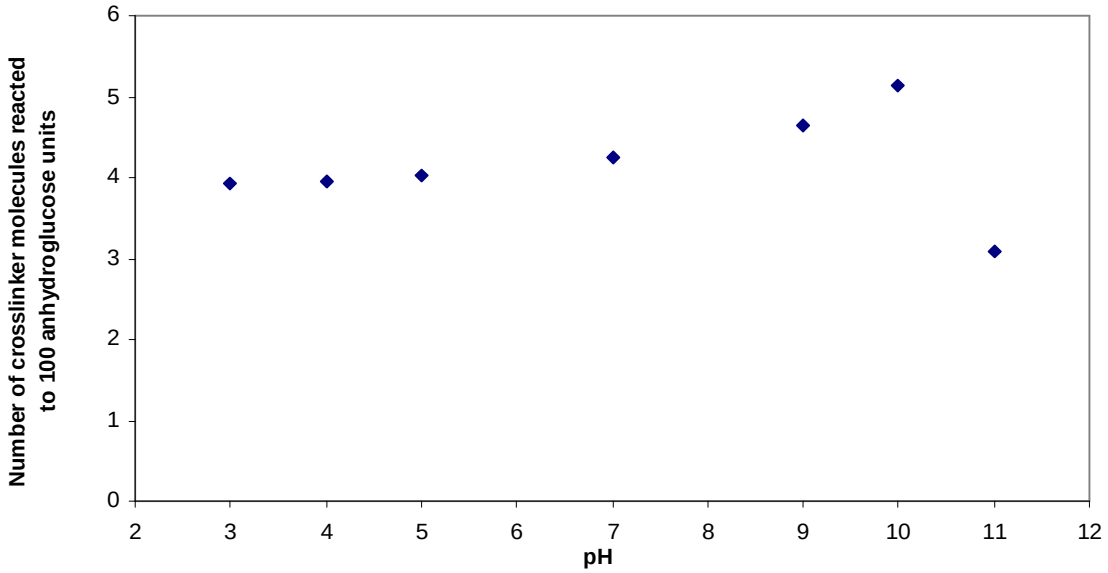


Figure 6. Effect of pH on degree of substitution with TMC as crosslinker and maltodextrin (MCS). Reaction was carried out for 24 hr.

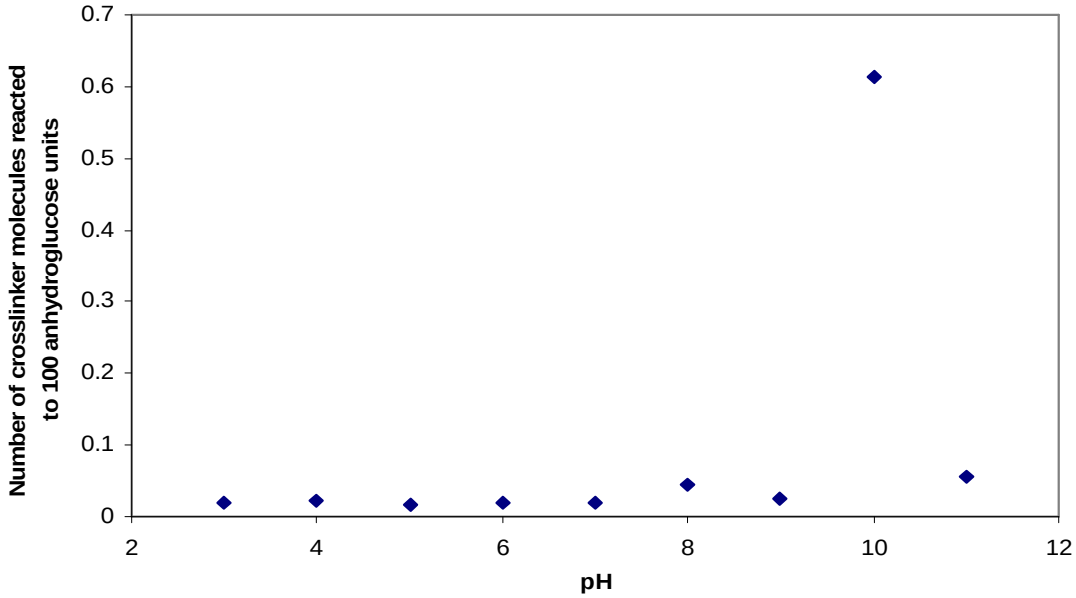


Figure 7. Effect of pH on degree of substitution with DEMC as crosslinker and DHPA. Reaction was carried out for 24 hr.

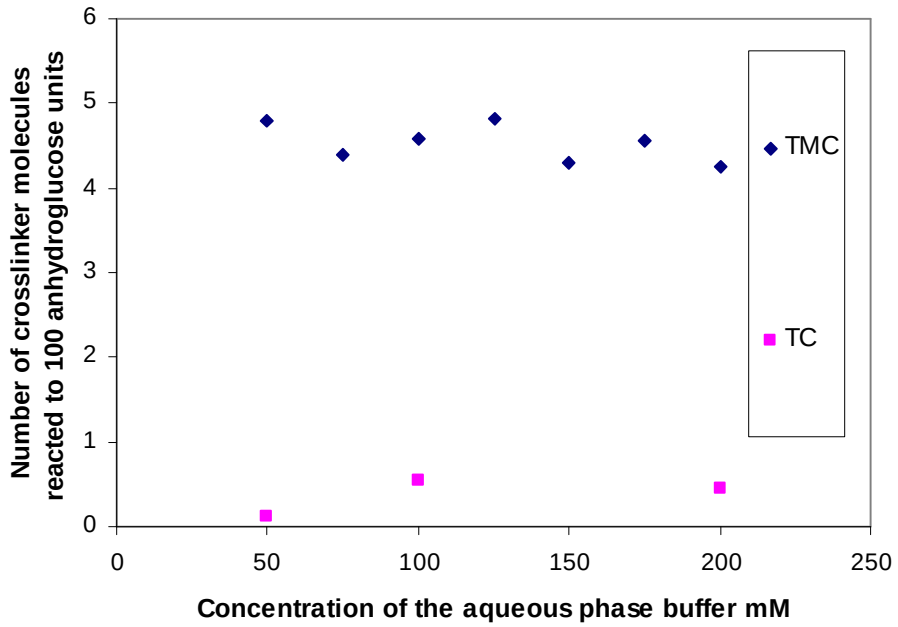


Figure 8. Effect of buffer strength on degree of crosslinking with TMC and TC as crosslinker and DHPA. The TMC batches were reacted for 24 hr at pH 7.0 while the batches with TC were reacted for 3 hr at pH 10.0.

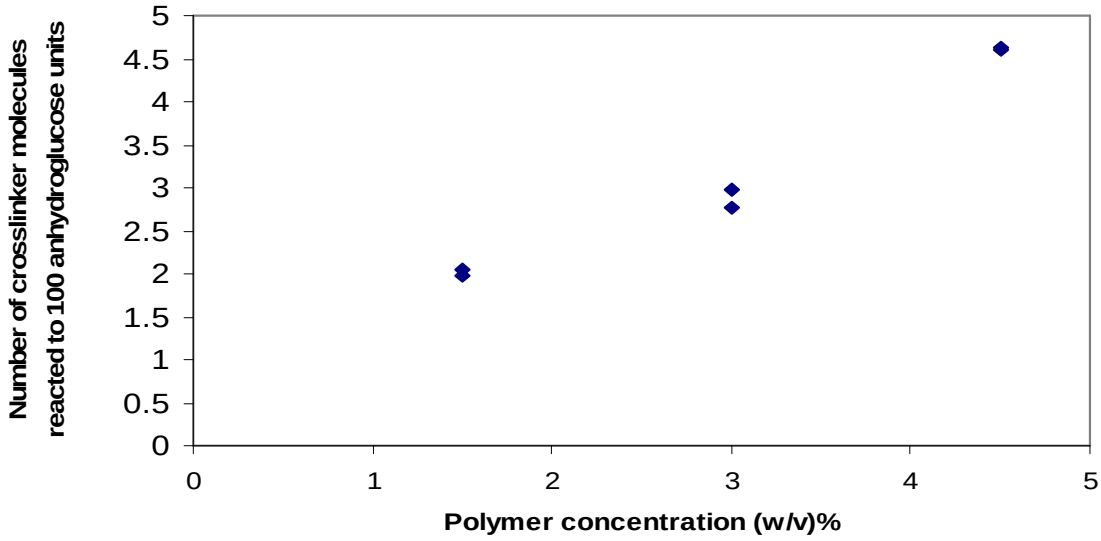


Figure 9. Effect of polymer concentration on degree of substitution with TMC as crosslinker and DHPA. Reaction was carried out for 24 hr at pH 7.0.

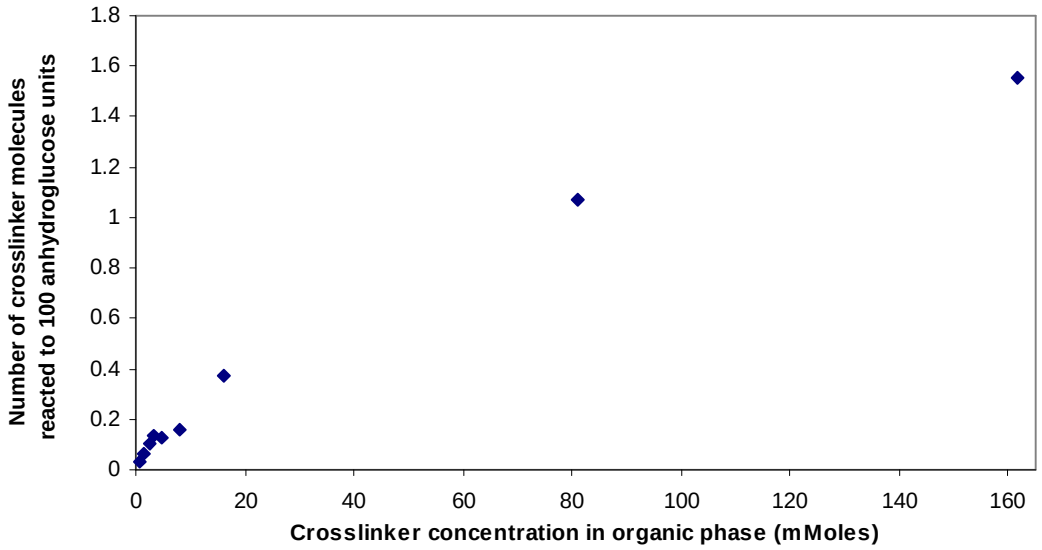


Figure 10. Effect of crosslinker concentration on the degree of substitution with TMC as crosslinker and MA as polymer. Reaction was carried out for 24 hr.

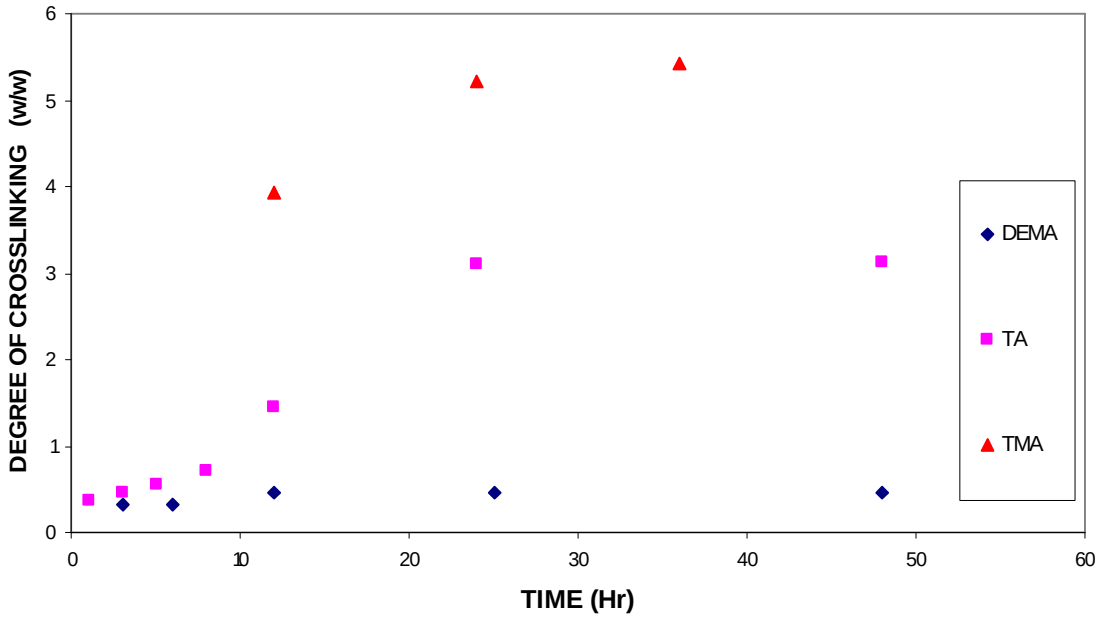


Figure 11. Effect of reaction time on the degree of substitution with TMC, TC and DEMC as crosslinkers and MCS as polymer and at pH 10.0.

V. INCOPORATION INTO AND RELEASE OF TARTRAZINE AND LYSOZYME FROM CARBOHYDRATE NANOPARTICLES

ABSTRACT

The preparation and characterization of carbohydrate nanoparticles with a hydrophilic surface has been reported elsewhere. In the present work, the suitability of nanoparticles as a sustained/controlled release platform for bioactive molecules will be investigated in detail. A model small molecule, tartrazine, and a protein, lysozyme, were incorporated into nanoparticles. Encapsulation efficiency and release profiles were determined for both molecules. The results show that the crosslinked membrane of the nanoparticles is not likely to produce adequate resistance to diffusional release of small molecules for most sustained release applications. However, the incorporation of small molecules into the nanoparticles revealed that high carbohydrate polymer load in the dispersed aqueous phase is necessary and sufficient to achieve high encapsulation efficiency of the incorporated molecules.

Pre-formulation studies with lysozyme revealed that when cyclohexane or chloroform was used as the continuum phase, high encapsulation efficiency was achieved. But when a combination of these two organic solvents or methylene chloride was used, encapsulation efficiency sharply decreased. Further, lysozyme encapsulation efficiency is strongly affected by pH of the dispersed aqueous phase. The optimum pH for encapsulation is 3.0. Carbohydrate polymer load also improves encapsulation efficiency.

Studies prove sustained release of lysozyme is achievable using this platform. Limited control over the release rate can also be exercised by varying the degree of substitution within a narrow range. Mathematical modeling of lysozyme release shows heterogeneous diffusion with two distinct modes, one slow and one very fast. The slower mode of diffusional release is believed to be the consequence of a diffusional barrier provided by the crosslinked membrane at the surface of the nanoparticles. The cause of the faster mode of diffusion in the initial period could not be definitely identified. Further research needs to be focused towards identifying and minimizing the initial burst release. Future research should also address the biologic disposition of nanoparticles specifically with regards to phagocytosis, *in vivo* release of bioactive molecules from the nanoparticles, particle integrity in physiological fluid, pharmacokinetic distribution and plasma half life of the nanoparticles.

1. INTRODUCTION

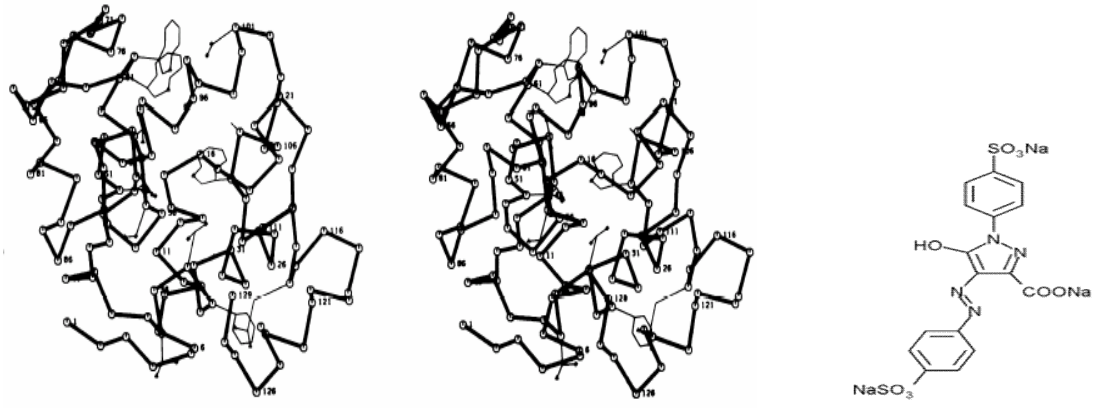
Carbohydrate polymers, both charged and uncharged have been used historically in drug delivery research to achieve controlled delivery. Charged carbohydrate polymers, like anionic alginates (Gombotz, W. R., 1998), pullulan (Mocanu, G., 2002), and cationic chitosan (Richardson, C. W. S., 1999; Mi, F. L., 1999) have all been used to produce microspheres as drug delivery platforms. The use of nonionic carbohydrate polymers like starch (Morath, L. P., 1998), high amylose starch (Lenaerts, V., 1998) and dextran (Mehvar, R., 2000; Stenkes, R. J. H., 2001; Franssen, O., 1999) for drug delivery has been gaining acceptance in recent years. Microspheres have been prepared with alginic acid by utilizing the ionic interactions between the carboxylic acid groups in the polymer

and di or trivalent metal ions like Ca^{+2} or Al^{+3}. The limitation of these systems is that the ionic interaction is reversible and the integrity of the microspheres are compromised in the presence of other ionic species like citrates, phosphates, Na^{+} and Mg^{+}, which are normally present in physiological fluids. The resulting disintegration of nanoparticles on exposure to physiological fluids causes rapid release of encapsulated bioactive molecules (Gombotz, W. R., 1998). Polyion condensation has been used to produce micro/nanoparticles with chitosan and DNA for potential use as non-viral DNA delivery vectors. Apart from the fact that all cationic polymers including chitosan possess considerable toxicity (Richardson, C. W. S., 1999), chitosan microspheres are also made through ionic interactions and thus suffer from similar limitations as alginates. A variety of different crosslinking agents have been used to produce micro/nanoparticles by crosslinking nonionic carbohydrate polymers throughout the device. Crosslinking in the bulk, especially with strong crosslinking agents like gluteraldehyde (Tabata, Y., 1989) is always associated with concerns of reacting the encapsulated bioactive compound itself and thus denaturing and/or immobilizing them in the particle.

In an attempt to address these concerns, carbohydrate nanoparticles have been prepared using a novel emulsion/interfacial crosslinking reaction. The main advantage of this platform is that the encapsulated bioactive molecules remain in a benign microenvironment at the core of the device unaffected by the interfacial crosslinking or other harsh preparative conditions. Another important aspect of this drug delivery system (DDS) is that being made up of carbohydrate polymers, this DDS has a hydrophilic surface which is expected to minimize if not eliminate the activation of the reticuloendothelial system and thus the removal of the particles from blood stream

through phagocytosis when introduced into the body. In addition, aggregation in aqueous supporting matrices is eliminated.

Having characterized the new carbohydrate nanoparticulate DDS in terms of physical and chemical properties, the object of the current research is to investigate complexities involved with the incorporation of potential bioactive molecules into the nanoparticles and their subsequent *in vitro* release from the particles in simulated physiological conditions. Two molecules have been chosen for the encapsulation to represent the two



1a. **1b.** **1c.**

Scheme-1. A schematic depiction of the two [(a) and (b)] natural stereo structures of lysozyme. Proc. Natl. Acad. Sci., USA, 1983, (80): 5773. 1(c) is a depiction of the molecular structure of tartrazine.

extreme ends of the spectrum of bioactive molecules. Tartrazine, a hydrophilic small molecule, containing strong anionic groups, is chosen to represent traditional small drug molecules [Scheme-1(c)]. Chicken egg white lysozyme is a moderately large protein (molecular weight 14,700) with native stereo structures [Scheme 1(a) and 1(b)]. It is a relatively hydrophobic protein carrying a net cationic charge at pH 9 and below and has been chosen to represent the emerging group of polypeptides and proteins that are

increasingly being used in the treatment of disease conditions. Lysozyme has a considerably large hydrodynamic diameter of 4.1 nm (Jensen, M., 2002) and slower diffusion of the molecule out of the nanoparticles is expected. The main goals pursued in this effort are to maximize encapsulation efficiency and quantitatively describe the mechanism of release.

2. EXPERIMENTAL

2.1 Materials

The carbohydrate polymers were procured from a variety of commercial sources and used without further purification. The polymers include corn starch (CS) (Sigma, St. Louis, MO), waxy maize starch (WMS) (PFP 2850, Cargill Foods, Minneapolis, MN), maltodextrin (MA) from waxy maize starch (C*Dry 01955, Cerestar USA Inc., Hammond, IA), and maltodextrin (MCS) from corn starch (Maltrin M40, Grain Processing Corporation, Mucatine, IA). The crosslinkers include terephthaloyl chloride (TC) (Sigma-Aldrich, St.-Louis, MO), and trimesoyl chloride (TMC) (Across organics, Pittsburg, PA). Other chemicals include sorbitan-mono-palmitate (Span-40), buffer salts (Sigma-Aldrich, St. Louis, MO), chloroform (CL), cyclohexane (CH), methylene chloride (MC), acetone, and ethanol (Fisher Scientific, Pittsburg, PA). All solvents were of ACS grade. Dimethyl sulfoxide (DMSO), acetonitrile (ACN), tetrahydrofuran (THF), and dimethyl formamide (DMF) were used as high pressure liquid chromatography (HPLC) solvents. Triethylamine (TEA), phenacyl bromide (PB), sodium azide (SA) are other reagents (Fisher Scientific).

Organic phase one (OP-1) was prepared by dissolving 6 % (w/v) Span 40 in a mixture of cyclohexane and chloroform (68/32, v/v) as the continuum phase of the emulsion. Organic phase two (OP-2) was prepared by dissolving 2.5 % of TC or 2.27 % (w/v) of TMC in cyclohexane and chloroform (68/32, v/v) for carrying out the crosslinking.

2.2 Preparation of nanoparticles and incorporation of tartrazine

Carbohydrate nanoparticles were prepared by an interfacial crosslinking reaction as earlier described. Tartrazine nanoparticles were made using combinations of CS, WMS and maltodextrins MCS and MA as polymers with TC and TMC as crosslinkers.

Briefly, the carbohydrate polymers CS, WMS, MCS and MA were heated in an appropriate volume of buffer in screw capped bottles to produce carbohydrate colloids in the case of high molecular weight carbohydrates, and true solutions in the case of maltodextrins. The concentration of carbohydrate polymers was maintained close to their maximum solubility (Table-1). The pH of the aqueous phase was maintained at 10.0 by using 200 mM borate buffer to maximize the encapsulation of tartrazine. The colloid or solution was allowed to cool to room temperature while stirring before tartrazine was incorporated at a concentration of 10% (w/w) relative to the polymer.

Twenty (20.0) mL of the carbohydrate colloid/solution was emulsified in 100 mL of OP-1 in a Silverson LR-4® homogenizer at 5,000 rpm for 1 minute. The emulsion was then poured into 100 mL of organic OP-2. The reaction was continued for 24 hr. The final concentration of both the crosslinkers was ~55 mM in the reaction mixture. The reaction was stopped by pouring the reaction mixture into 700 mL of ethanol in a 1000 mL beaker while stirring. The precipitated nanoparticles were washed 3 times with

ethanol and 3 times with acetone by repeated centrifugation at 1,500 g and vortexing following discontinuation of stirring. Finally, the suspension of nanoparticles in acetone was dried under vacuum at 14.5 psi at room temperature (20-25°C) overnight.

2.3 Encapsulation and release studies with tartrazine

To evaluate the effect of degree of substitution (DS) on the release of tartrazine the following protocol was observed. Two hundred and twenty (220) mg of dry nanoparticles with a theoretical loading of 9.09% (w/w) of tartrazine was dispersed in 5 mL of 100 mM potassium phosphate buffered saline (KBPS) at pH 7.4. The suspension was immediately placed into an 18 cm long dialysis membrane tube (SpectraPor®, Spectrum Laboratories Inc., Ranco Dominguez, CA; molecular weight cutoff of 50,000 Daltons). One end of the dialysis tube was clamped securely, the suspension added and the other end was clamped such that the distance between the two clamps was 5 cm in each case. The lower clamps had a magnetic weight built in so that the formed pouch floats upright and remains stable during stirring. The pouch containing the suspended nanoparticles was placed in a 1000 mL beaker containing 495 mL of 100 mM KBPS. To minimize evaporation of the dissolution media, the beakers were covered with a thin polyethylene sheet (Clingwrap®). A multiple head magnetic stirrer with precise control was used to maintain a stirring speed of 150 rpm. One (1.0) mL samples were withdrawn at predetermined time intervals and the media replenished each time. Tartrazine was quantified by absorbance spectroscopy using a Beckman UV/VIS spectrophotometer at 445 nm wavelength. To account for any possible contribution of the dialysis membrane to tartrazine delivery, 20 mg of tartrazine was dissolved in 5 mL of 100 mM KPBS (4

mg/mL). The aliquot was placed into dialysis tubing as previously described and used as the control.

At the end of the release studies, the asymptotic level of tartrazine concentration was used for encapsulation efficiency based on: $E = (A/T)*100$, where E is the encapsulation efficiency, A is the actual amount of tartrazine released and T is the amount (9.09 %) of tartrazine added.

2.4 Partitioning and interfacial aggregation of lysozyme

In order to ensure maximum loading of lysozyme in the carbohydrate nanoparticles, pre formulation studies were performed. The effect of organic solvents used as the continuum phase, pH, and carbohydrate load of the dispersed aqueous phase on lysozyme partitioning was investigated. Firstly, 4.5 % (w/v) lysozyme was dissolved in deionized water. The pH of the deionized water was adjusted to 3.0, 4.0 and 5.0 by adding 50, 20, 10 μL of 10 % (v/v) trifluoroacetic acid. The final concentration of trifluoroacetic acid was 1.345, 0.054 and 0.027 mM, respectively. Since lysozyme is a basic protein with an isoelectric pH of 11.0 (Alderton, G., 1945), the series of pHs chosen are expected to fully ionize the amino groups resulting in minimum partitioning into the continuum phase. Twenty (20) mL of this aqueous phase was homogenized with 100 mL of methylene chloride, cyclohexane, chloroform or cyclohexane/chloroform (68/32, v/v). Surfactant was deliberately omitted from the preformulation study to ensure adequate separation of the phases. The homogenized phase was allowed to separate on standing overnight at room temperature. After the phases separated, 1.0 mL samples were collected from the

aqueous phase, centrifuged at 1,500 g to remove any insoluble aggregated lysozyme and analyzed by HPLC.

2.5 Incorporation of lysozyme in nanoparticles

Lysozyme was incorporated in nanoparticles made from maltodextrin obtained from waxy maize starch (MA). The crosslinker used was TMC. The preparation procedure used was similar to the process already described. Only the modifications will be mentioned in this section. Forty five (45) percent (w/v) of MA was dissolved in the aqueous phase buffered at pH 3.0 by heating. Unless stated otherwise, 4.5 g of lysozyme was added to 50 mL of MA solution upon cooling to room temperature. The solution was slowly stirred until the lysozyme dissolved. A final theoretical loading of 9.09 % (w/w) was obtained. Nine (9) g of lysozyme was added to the same volume of MA solution in some batches to achieve a theoretical lysozyme loading of 16.67 % (w/w). The TMC concentration was varied from 0.8 to 486 mM in the continuum phase.

2.6 Lysozyme assays

2.6.1 Assay of lysozyme from release samples

Standard protein solutions were made in the concentrations of 1, 5, 10, 20, 30 and 40 μg/ml in 100 mM (KPBS) containing 0.2 % (w/v) sodium azide. To account for any interference from the nanoparticles in authentic samples, blank nanoparticles were suspended in the standard solution in the same concentration used in the release experiments. As a low molecular weight carbohydrate scavenger, 75 μL of n-butanol was added to 1.0 mL of the sample in a 1.5 mL polypropylene micro centrifuge tube.

The samples were then centrifuged at 14,000 g for 15 min to separate any precipitated carbohydrate residue and the suspended nanoparticles. Approximately 250 μL of the supernatant was then transferred to inserts in autosampler vials. Seventy (70) μL of the sample was injected for HPLC analysis. Both the lysozyme standards and samples from release studies were identical to one another in composition except for the lysozyme content.

The stationery phase was a Zorbax SB300® column with 5 μ particle size, 300 Å pore size, C_{18} packing, and with 4.6 x 150 mm column dimension. A 4.6 x 10 mm precolumn of the same stationary phase composition was used as a protective device. The mobile phase was composed of 57 % (v/v) of solvent A and 43 % (v/v) solvent B pumped at a flow rate of 1.0 mL/min. Solvent A was composed of deionized water containing 0.1 % (v/v) trifluoroacetic acid (TFA). Solvent B was composed of 4 % (v/v) DMSO, 20 % (v/v) THF, 76 % (v/v) ACN, and 0.1 % (v/v) TFA.

The chromatography was carried out at ambient temperature. The chromatographic system consisted of a Thermo Separation (San Jose CA, USA) Model P2000 pump used in the isocratic mode, a Model AS3000 autoinjector, and a Model FL2000 variable-wavelength fluorimetric detector. The detection was carried out at 280 nm excitation and 340 nm emission wavelengths. Lysozyme eluted at 4.2 min. The calibration curve was constructed in the concentration range of 1 - 40 μg/ml and all release study measurements were conducted within the range.

2.6.2 Content assay of lysozyme

For quantifying nanoparticle lysozyme content, a slight modification of the HPLC sample preparation method was adopted for optimum efficiency. An appropriate amount of dry nanoparticles were dispersed in 1.0 mL of 100 mM KPBS containing 0.2 % (v/v) SA. The suspension was diluted with 2.0 mL of deionized water and 1.0 mL of mobile phase, mixed for 2 min in a vortex mixer, and kept for 1 hr at room temperature. A 70 μL aliquot was injected. The DMSO present in the mobile phase was found to dissolve the nanoparticles. A calibration curve was constructed and used for this purpose in the range of 1-40 μg/ml. The sample amount of the nanoparticles was adjusted to maintain lysozyme concentrations within the calibration range.

2.7 Crosslinker assay

The degree of substitution of TC and TMC were measured by a reverse phase HPLC method (Basu Sarkar, A., 2005).

2.8 Release studies with lysozyme

Lysozyme release was evaluated from a dispersion of nanoparticles at pH 7.4 in 100 mM KPBS. The dialysis membrane was omitted since prolonged mass transfer was observed for non-encapsulated lysozyme solution using a 100,000 Da molecular weight cutoff dialysis membrane. Two hundred and twenty (220) mg of dry nanoparticles containing lysozyme in the concentrations of either 9.09 or 16.67 % (w/w) were dispersed in 500 mL of 100 mMolar KPBS containing 0.02 % (w/v) of SA in a 1000 mL conical flask. To avoid evaporation of the aqueous media, the flask was covered with a

thin polyethylene sheet (Clingwrap®). Sink conditions were maintained by a volume of 500 mL of reslese media and stirring the media at 150 rpm. One (1.0) mL of sample was withdrawn at predetermined time points for quantification. The samples were handled as described in the lysozyme assay and 70 μL of the sample was injected for analysis by HPLC.

2.9 Regression analysis and model assessment for lysozyme release

Nonlinear least squares regression was used to analyze the underlying mechanism of lysozyme release based on a spherical diffusion model. Initially a homogeneous diffusion model (Kim, C., 2000) was used as described by the following equation (1).

$$\frac{M_t}{M_\infty} = 1 - \frac{6}{p^2}\sum_{n=1}^{\infty}\frac{1}{n^2}e^{-Pn^2t} \quad P = \frac{Dp^2}{r^2} \qquad (1)$$

Here, 'M_t' is the mass of lysozyme released in time 't', 'M_∞' is the mass of lysozyme released at infinite time, 'n' is the summation index varying from one to infinity, 'P' is the permeability coefficient, 'D' is the diffusion coefficient of lysozyme, 'r' is the mean radii of the spheres and the term M_t/M_∞, denotes fractional release of lysozyme at time 't'. Best fit statistical analysis demonstrated the discrimination of two independent modes of lysozyme diffusional release. As a consequence, a heterogeneous diffusional model was adopted for subsequent analysis as described by equation (2).

$$\frac{M_t}{M_\infty} = \frac{M_{1,\infty}}{M_\infty}\left(1 - \frac{6}{p^2}\sum_{n=1}^{\infty}\frac{1}{n^2}e^{-P_1 n^2 t}\right) + \frac{M_{2,\infty}}{M_\infty}\left(1 - \frac{6}{p^2}\sum_{n=1}^{\infty}\frac{1}{n^2}e^{-P_2 n^2 t}\right)$$

$$P_1 = \frac{D_1 p^2}{r^2}\ ,\ P_2 = \frac{D_2 p^2}{r^2} \qquad (2)$$

Here, $M_{1,\infty}$ is the mass of lysozyme released by mode 1 at infinite time, $M_{2,\infty}$ denotes the mass of lysozyme released by mode 2 and M_∞ is the total mass of lysozyme released.

In both models, a ten-term truncation of the infinites series was found adequate for data analysis. The series coefficient ($6/\pi^2$) was appropriately adjusted to a value of 0.64526 corresponding to the reciprocal sum of the first ten terms of the series at t = 0.

All curves were analyzed using normalized data where M_∞ was estimated from the numerical average of the last 2-3 asymptotic data values. Residual sum of squares was weighted using a factor of 1.0 for regression analysis. The parametric values of P_1, P_2, f_1, and f_2 were estimated, where $f_1 = M_{1,\infty}/M_\infty$ and $f_2 = M_{2,\infty}/M_\infty$.

3. RESULTS

3.1 Encapsulation and release of tartrazine: The effect of polymers

Tartrazine was incorporated in nanoparticles synthesized from the carbohydrate polymers CS and WMS and the oligomers MCS and MA using both TC and TMC as crosslinkers. Variable encapsulation efficiency was observed (Table 1). Nanoparticle batches prepared from carbohydrate polymers CS and WMS showed relatively low

encapsulation efficiency of 32 to 39 % of the incorporated tartrazine irrespective of the crosslinker used. Batches made from carbohydrate oligomers MCS and MA showed higher encapsulation efficiencies of 73 and 84 %, respectively.

Following completion of the crosslinking reaction, the dispersed aqueous phase was extracted in a large volume of ethanol resulting in a rapid precipitation of the polymer. Though tartrazine is not soluble in any of the solvents, cyclohexane, chloroform or ethanol, it is extractable from the nanoparticles in the presence of small amounts of water provided by the dispersed phase. The small amount of tartrazine remained in the nanoparticles prepared from CS and WMS because it was entrapped in the rapidly precipitating polymers. The entrapment effect of the encapsulated tartrazine was low for CS and WMS because of the low polymer loads that can be achieved with these polymers in the dispersed phase. In cases of MCS and MA, the entrapment effect of the carbohydrate is much more pronounced due to a ten fold higher carbohydrate load [45-50 % (w/v)], made possible by their higher aqueous solubility.

Release studies from TC (Figure 1) batches showed significant sustained release from the branched polymers WMS and MA as compared to the control. Nanoparticles made from the other two polymer species, CS and MCS, showed very little difference in their release profiles compared to the control. Release studies from TMC (Figure 2) shows significant sustained release of tartrazine only from the nanoparticles made with WMS. A comparison (Figure 3) of the tartrazine release from nanoparticles synthesized with TC and TMC shows that the branched polymer amylopectin (WMS) imparts more sustained release than regular corn starch for both the crosslinkers.

Although some differences in tartrazine release were observed relative to the specific carbohydrate used for encapsulation, comparative tartrazine delivery relative to the control was unremarkable. These results indicate that the encapsulating membrane offers little resistance to tartrazine diffusional mass transfer.

3.2 Lysozyme assay

3.2.1 Accuracy and precision of release samples assay

The accuracy of lysozyme determinations (Table 2) ranged from 96 to 103 % corresponding to nominal concentrations of 1.0 to 40 μg/mL. The precision as described by the relative standard deviation (RSD) was found to be < 2.5 % corresponding to concentrations from 5 to 40 μg/mL. At the 1 μg/mL concentration level, the precision was approximately 20 % of the mean. This concentration level was considered the lower limit of detection. Chromatography was stable and no distortion of peak shape or deviation of retention time was observed between standard and actual samples (data not shown).

3.2.2 Accuracy and precision of the content assay

In the content assay, the accuracy of lysozyme determinations (Table 3) varied from 90 to 110 % for concentrations ranging from 1 to 40 μg/mL. The precision, as described by the relative standard deviation was between 1 to 3.5 % for every concentration level except 1 μg/mL which is considered the lower limit of quantification for this assay as well. At this level, the precision was 24 % of the mean. The chromatography was less stable compared to the release sample assay of lysozyme. Some broadening of the peak

was observed though retention time did not change. Peak height was used instead of peak area for constructing calibration curves.

3.3 Partitioning of lysozyme and its encapsulation efficiency

The results of lysozyme partitioning experiments using methylene chloride, cyclohexane, chloroform and cyclohexane/chloroform [68/32, (v/v)] at pHs 3.0, 4.0, and 5.0, showed that lysozyme partitioning depended heavily upon the exact set of conditions. When MC was used as the continuum phase (Table 4), no lysozyme was detected in the aqueous phase following emulsification and phase separation at any pH tested. Insoluble aggregated lysozyme was observed at the interface and also in both phases. Lysozyme partitioning into the organic phase and aggregation at the interface were found to be low when CH and CL were used separately as the organic phase. The soluble lysozyme, recovered from the aqueous phase following emulsification and phase separation, were 73 and 75 % for CH and CL, respectively, at pH 3.0. However recovery of soluble lysozyme in the aqueous phase was 10 % when CH/CL [68/32, (v/v) %] was used as the continuum phase at pH 3.0. Also, the percentage of soluble lysozyme decreased rapidly with the increase in pH in all cases in which lysozyme was detected in the aqueous phase. The observation of progressively lower recovery of lysozyme from dispersed phase as the pH increased could be attributed to the higher degree of protonation of the amino groups of lysozyme. Thus, for incorporation of lysozyme, CL was selected as the continuum organic phase and the pH of the dispersed aqueous phase was selected to be 3.0. Lysozyme is known to be stable for several weeks at low acidic pH.

In order to investigate the effect of carbohydrate content on lysozyme partitioning, studies were conducted with 15, 30 and 45 % (w/v) of MA in the aqueous dispersed phase at pH 3.0 with CL as the organic continuum phase. The results show a near quantitative recovery of soluble lysozyme in the aqueous phase following emulsification and phase separation with all levels of carbohydrate in contrast to 75 % recovery from the identical phase system without any carbohydrate (Table 5). These results lead to the conclusion that high carbohydrate load cause lysozyme retention in the dispersed phase due to carbohydrate protein interaction. Table 6 shows the actual lysozyme encapsulation efficiency measured in representative batches. The encapsulation efficiency averaged 98 ± 10.2 % of the added lysozyme.

3.4 Effect of lysozyme loading on degree of substitution

The effect of lysozyme loading on the DS was assessed with two different levels of lysozyme loading with varying TMC concentrations. The results (Figure 4) show that the DS increases with TMC concentration in the continuum phase as expected in both cases. The DS is hyperbolically dependent on TMC concentration. The observed differences in reactivity were associated with lysozyme load when the TMC concentration exceeded 16 mM. This observation suggests the presence of lysozyme at the interface and excessively large protein loads can interfere with interfacial crosslinking. This effect probably limits protein loading in encapsulated systems.

3.5 The effect of crosslinking on lysozyme release

Nanoparticles were prepared with the same lysozyme loading [9.09 % (w/w) of the dry nanoparticles] and 5 different levels of TMC substitution. The crosslinking was varied in terms of DS from 0.03 to 7.09 by varying the initial crosslinker concentration in the continuum phase from 0.8 mM for to 485.2 mM. The release studies show (Figure 5) that DS has a profound effect on the extent of lysozyme release as discussed later.

Nanoparticles with a DS of 0.03 showed instantaneous release of the encapsulated lysozyme. The resistance effect of the crosslinked membrane was completely absent. Nanoparticles with DS of 0.13 to 0.16 released 27 and 18 % of encapsulated lysozyme in the first hour, 48 and 26 % after 2 days, and 62 and 39 % in 11 days, respectively, and no significant release thereafter in the time frame of the release studies. Continuous and sustained release characteristics of the nanoparticles are expressed prominently in this range of DS. Nanoparticles with DS between 0.03 and 0.13 showed some sustained release for up to 2 days (data not shown). Further increase of DS to 1.07 resulted in very slow release of ~13 % in 1 hr and up to ~20 % in two days and approximately 22, 23 and 24 % release on days 10, 12 and 15 respectively. For DS of 7.09 lysozyme release virtually stopped with 3 % in 1 hr, ~ 8 % in 2 days and no significant release thereafter.

An inverse relationship between the extent of lysozyme released in 15 days and the DS of TMC was evident (Figure 6). As the DS increased from 0.032 to 7.094, the extent of lysozyme release decreased rapidly up to a DS value of 0.158. The extent of lysozyme release decreased slowly thereafter up to a DS of 7.094.

It is apparent that there is a narrow range of substitution in which the lysozyme release can be controlled. This range corresponds with DS values between 0.13 and 1.07.

Another significant observation is that the varied degree of crosslinking affects the extent of lysozyme release more significantly than the rate of release.

3.6 Modeling of lysozyme release

A homogeneous diffusion model initially used to mechanistically explain lysozyme release from nanoparticles was found to be inadequate (Figure 7). As evident from the figure, the errors or residuals from the predicted data are not random but systematic. The residuals progressively decrease from a positive value to negative as time increases. The same trend was also observed with the release data from other representative batches (data not showed).

In an effort to fully capture the complexity of the events, a heterogeneous diffusion model was then applied for data analysis. Resulting fitted curves (Figure 8-11) all show high correlation with coefficients (r^2) > 0.985 and random residuals, thereby validating the model.

Figure 12a and 12b show the calculated release profiles of lysozyme from nanoparticles (DS 0.132) in the time frames of 24 hr and 12 days. The profiles in each figure correspond to the two individual diffusional modes of release characterized by P_1 and P_2. The first mode, characterized by P_1, is very fast and is complete by approximately three hours in all cases (data not shown). The second mode, characterized by P_2, is slower. The diffusional release due to the second mode continues for approximately two weeks.

The permeability coefficient P_2, corresponding to the slower mode of lysozyme release, was found to be related to the DS (Figure 13). This finding suggests that the

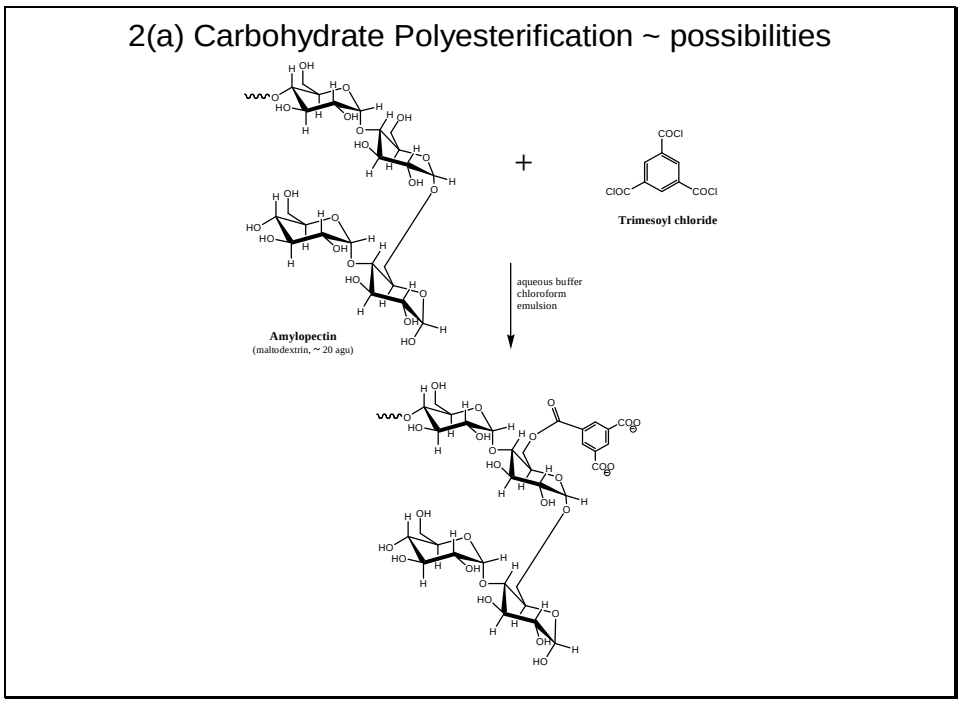
slower mode of lysozyme release may be due to the resistance provided by the polymerized membrane at the nanoparticle surface. No such relationship was found with P_1.

3.7 The effect of lysozyme loading on the release of lysozyme

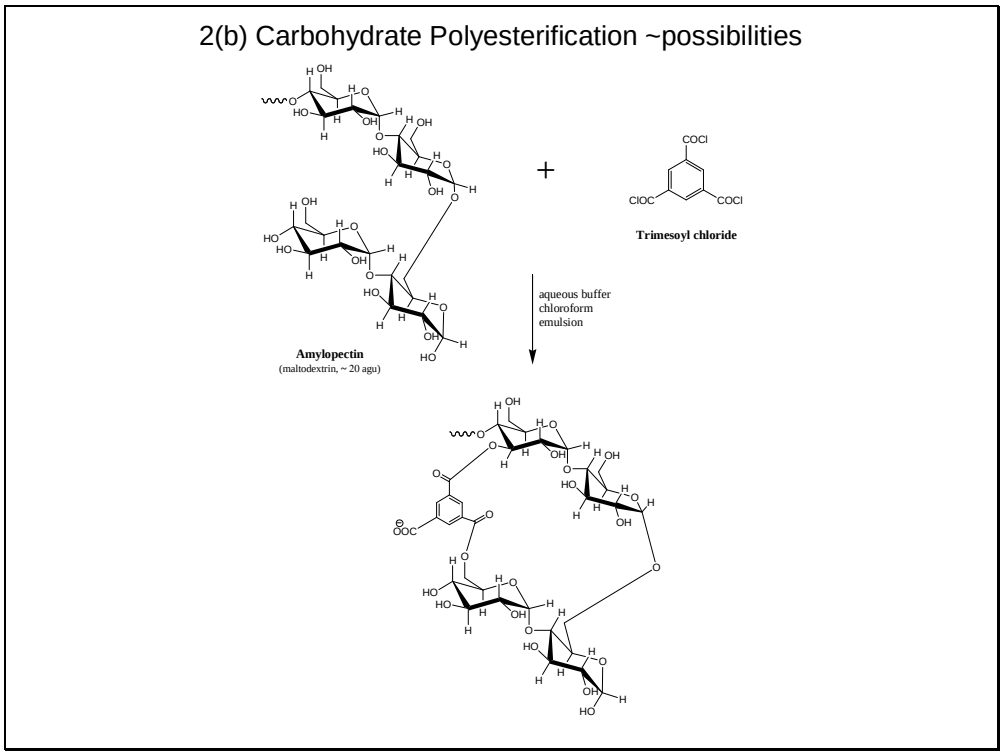
Nanoparticles with lysozyme loading of 9.09 % (w/w) and 16.67 % (w/w) were prepared under identical conditions of crosslinking. The release profiles showed (Figure 14) that lysozyme release from nanoparticles with 16.67 % loading was much faster (P_2 0.371) and more complete than from the nanoparticles with lower lysozyme loading of 9.09 % (P_2 0.198).

4. DISCUSSION

Crosslinking of carbohydrate polymers with the tri functional TMC reagent at the emulsion interface is an exceedingly complicated reaction. Many different products are possible. Mono, di or tri esterification as well as inter chain crosslinking, intra chain crosslinking, and chain elongation of the carbohydrate polymers are all possible. A crosslinking reaction involving at least two acid chloride groups is the goal of the interfacial esterification reaction. But it is dependent upon the availability of alcoholic hydroxyl groups of the carbohydrate polymers in the right spatial arrangement with respect to the crosslinker molecule. The hydroxyl groups of the carbohydrate polymers have to be present at the interface in sufficient concentrations so that the groups are available for reaction.



Scheme 2. A schematic representation of some of the possibilities of the carbohydrate crosslinking with trimesoyl chloride. 2(a) shows a mono esterified trimesic acid with two free acid groups. 2(b) a di esterified trimesic acid with only one free acid group.



When lysozyme is incorporated into the particles, an amide formation as a result of the reaction between acid chloride groups of the crosslinker and free amino groups of the protein is also possible. This side reaction may also be aided by the fact that lysozyme, like most moderate sized proteins, has some surfactant activity and tends to migrate to the interface. It is expected that the polymerization of lysozyme into the carbohydrate polymer would reduce the cumulative extent of lysozyme delivered, but not necessarily affect the superficial membrane since the carbohydrate is present in concentrations several magnitudes greater than lysozyme. It was also observed that when the lysozyme loading was increased a more complete release was achieved. The partitioning of lysozyme is a saturable phenomenon for a fixed surface area and thus the above observation can be readily rationalized. As the lysozyme concentration in the dispersed aqueous phase increased, a lower fraction of the incorporated molecule partitioned into the interface.

Mechanistic studies on lysozyme release demonstrated two modes of delivery, one relatively slow lasting about 2 weeks, and a fast mode which was complete within 2-3 hr. Results showed that P_2, the permeability coefficient related to the slower mode of lysozyme release, was correlated with the degree of substitution (DS). P_2 decreased with increase of DS indicating a dependence on crosslinking density. These results indicate that the slower mode is related to trans-membrane flux of lysozyme from the nanoparticle core load.

One observation of interest is that the initial extent of release varied widely. One hundred (100) % of the encapsulated lysozyme was released in 1 hr for the minimally crosslinked nanoparticles while for the nanoparticles with highest crosslinking the release

was only 3.5 % for the same time frame. It is well known that all starch polymers (Fundueanu, G., 2004) and dextrans (Denizli, B. K., 2004) swell on exposure to water. It is thus possible that the initial swelling on being introduced to the release medium was associated with relatively fast release of short duration. Another possibility is the fast dissolution and release of surface associated lysozyme. It is to be noted that at the end of the reaction, water from the dispersed phase was extracted by the introduction of a large volume of ethanol. During the extraction of water from the dispersed phase, migration of lysozyme to the surface is a possibility. The surface of the nanoparticles is potentially anionic as a result of the mono or di esterified TMC. Thus any superficial cationic lysozyme can remain electro statically associated with the anionic surface. This surface associated lysozyme can then be quickly released in simulated physiological fluid, resulting in the initial fast mode of release.

5. CONCLUSION

Several conclusions can be arrived at from the results of the present work. Firstly, both hydrophilic small molecules like tartrazine and moderate sized proteins can be successfully incorporated into the carbohydrate nanoparticles developed. For the small molecules, the barrier function of the crosslinked membrane at the interface is not adequate to achieve sustained release for most applications. For larger protein molecules like lysozyme with a hydrodynamic diameter 4.1 nm, the membrane is adequate to result in a significant sustained release of up to at least two weeks. Further work is needed to positively identify the mechanism of faster release associated with the observed delivery profile. Reduction in the extent of protein release during the first 2 hr period would be

desirable for sustained release applications, but not necessarily a requirement for pharmacokinetic distribution and cellular interaction. Lastly, though the hydrophilic carbohydrate nanoparticles are theoretically ideally suited to evade the opsonization process and their subsequent elimination from the blood stream, *in vivo* confirmation is needed to establish the integrity, half life of circulation and also the release pattern of encapsulated bioactive macromolecules from this platform. The physiological distribution of the prepared nanoparticles needs to be investigated in detail to unravel the entire spectrum of patho-physiological situations in which this platform can be a useful tool for medical intervention.

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Table 1. Effect of carbohydrate polymer on the encapsulation efficiency of tartrazine.

Crosslinker Concentration (w/v)%	Polymer Concentration (w/v)%	Encapsulation Efficiency of Tartrazine
TMC (1.635)	Corn Starch (4)	39.09
TMC (1.635)	Waxy maize starch (5)	32.43
TC (1.25)	Corn starch (4)	39.09
TC (1.25)	Waxy maize starch (5)	39.6
TC (1.25)	MCS (50)	78.36
TC (1.25)	MA (45)	83.89

Table 2. Accuracy and precision for the determination of lysozyme released from carbohydrate nanoparticles.

Calibration level (μg/ml)	Assay mean[a] (μg/ml)	SD	RSD (%)	Accuracy (%)
1	1.02	0.20	19.95	101.56
5	4.78	0.09	1.83	95.67
10	10.10	0.22	2.16	100.98
20	20.70	0.11	0.55	103.48
30	30.50	0.15	0.49	101.67
40	38.84	0.23	0.60	97.09

SD, standard deviation; RSD, relative standard deviation. [a] $N = 5$.

Table 3. Accuracy and precision for the determination of lysozyme in encapsulation efficiency studies of carbohydrate nanoparticles.

Calibration level (μg/ml)	Assay mean[a] (μg/ml)	SD	RSD (%)	Accuracy (%)
1	0.96	0.229	24.03	95.68
5	4.98	0.064	1.29	99.59
10	11.01	0.379	3.44	110.13
20	21.49	0.033	0.15	107.43
30	29.65	0.542	1.82	98.83
40	35.97	0.393	1.09	89.93

SD, standard deviation; RSD, relative standard deviation. [a] $N = 3$.

Table 4. Effect of pH and organic phase solvent on the partitioning behavior of lysozyme in the pH range of 3.0-5.0. Water-organic phase ratio was 1:5.

Solvent	Percent of total lysozyme found in aqueous phase at different pHs		
	pH 3.0	pH 4.0	pH 5.0
Methylene chloride	*ND	ND	ND
Cyclohexane	73.26	44.08	37.4
Chloroform	74.9	21.26	14.03
Cyclohexane: Chloroform [68:32, (v/v)]	9.95	7.16	4.69

*ND, not detected.

Table 5. Effect of carbohydrate content on the partitioning behavior of lysozyme at pH 3.0. The organic phase consisted of chloroform. Water/organic phase ratio was 1:5.

Aqueous phase description	Lysozyme remaining in aqueous phase (%)
0% (w/v) polymer (MA)	74.9
15% (w/v) polymer (MA)	100
30% (w/v) polymer (MA)	97.3
45% (w/v) polymer (MA)	95.3

Table 6. Encapsulation efficiency and degree of substitution achieved in some representative nanoparticle batches.

Batch #	Encapsulation efficiency	Degree of substitution*
NC-127	91.24	7.09
NC-136	95.36	0.159
NC-138	111	0.133
NC-139	108.97	0.128
NC-141	84.99	0.106
NC-142	96.26	0.059

*Degree of substitution refers to the number of crosslinker molecules reacted to 100 anhydroglucose units.

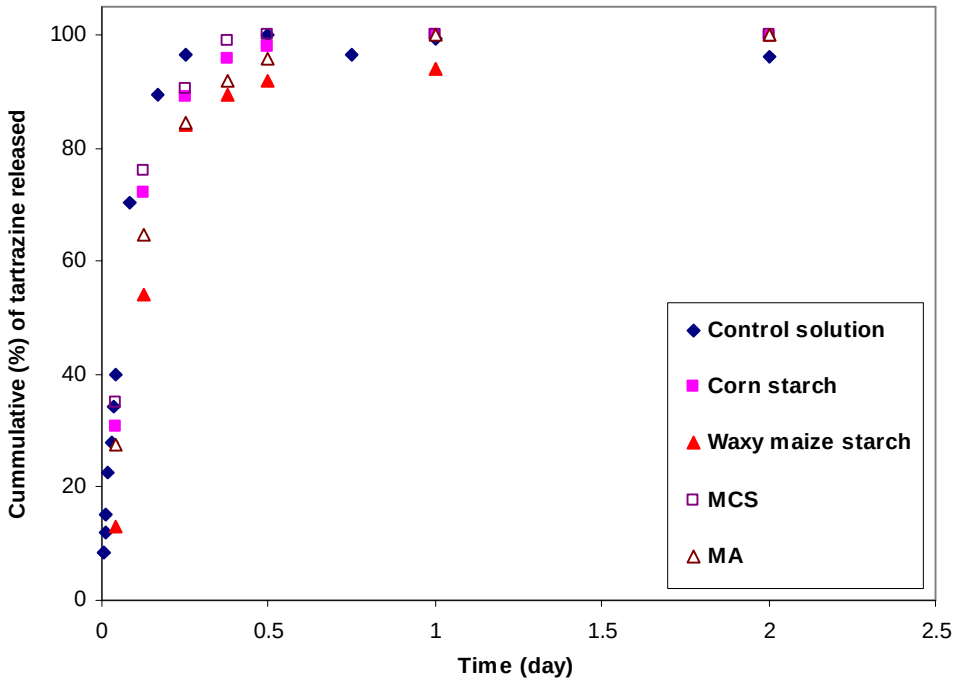

Figure 1. Comparative release profiles of tartrazine from nanoparticles made with TC as crosslinker.

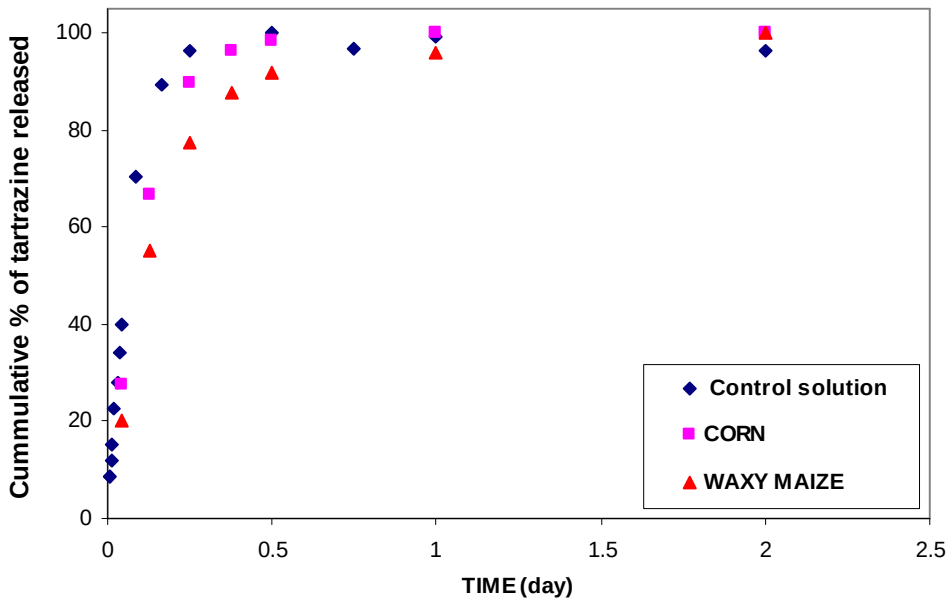

Figure 2. Comparative release profiles of tartrazine from nanoparticles made with TMC as cross linker.

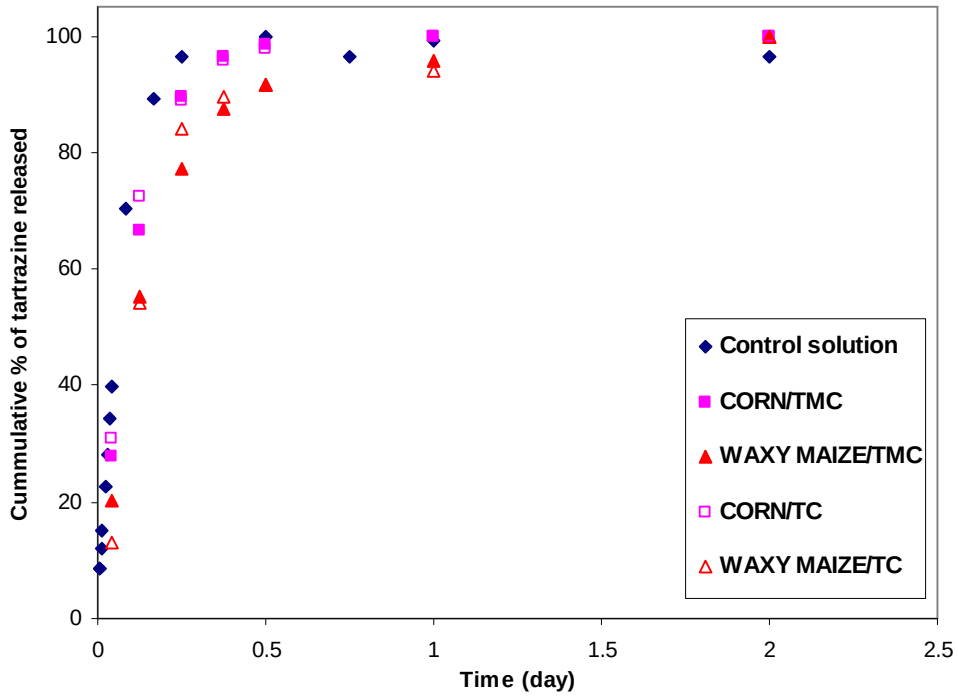

Figure 3. Comparative release profiles of tartrazine from nanoparticles made with TC & TMC as crosslinkers.

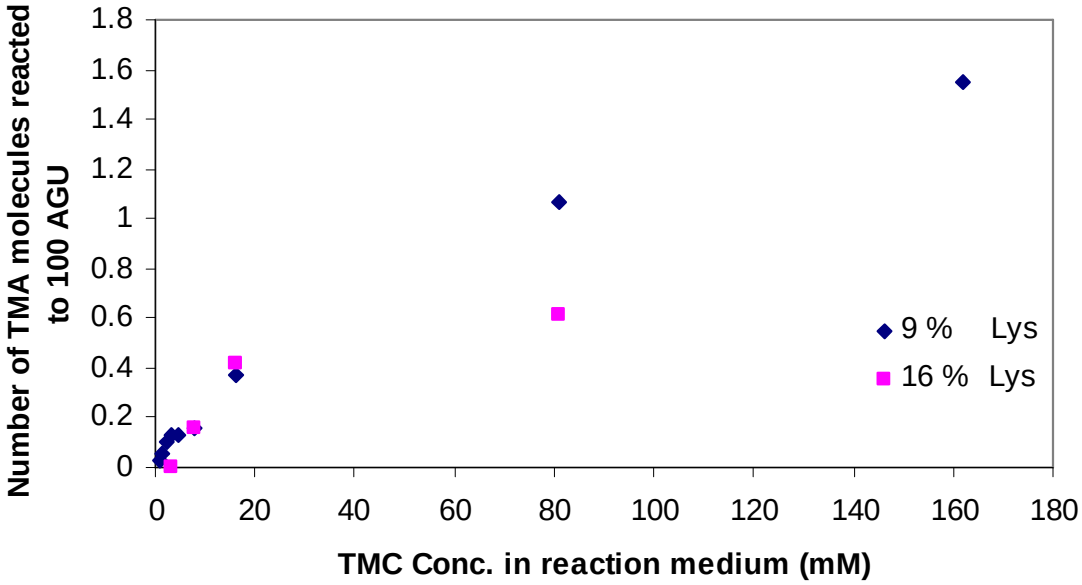

Figure 4. Relationship between degree of substitution (DS) and trimesoyl chloride (TMC) concentration in the continuum phase. The effect of lysozyme concentration on the degree of substitution is demonstrated at 9 and 16 % protein load.

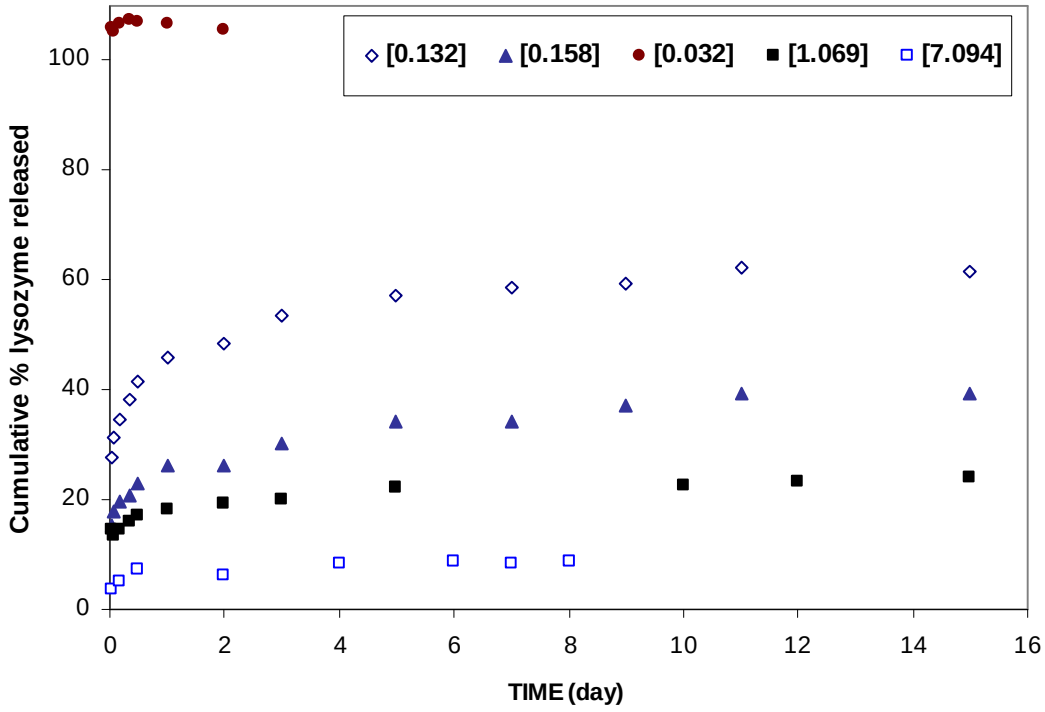

Figure 5. Comparative release profiles of lysozyme from nanoparticle batches with different TMC concentrations. The number in parenthesis indicates degree of substitution achieved in terms of number of trimesoyl chloride molecules reacted per 100 anhydroglucose units of the polymer.

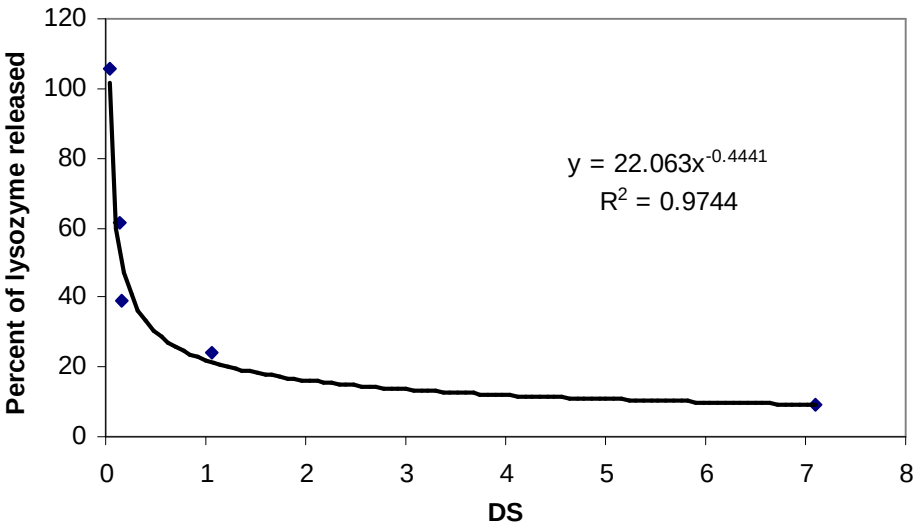

Figure 6. The relationship between the degree of substitution (DS) and the extent of release of lysozyme in 15 days from nanoparticles with different levels of crosslinking.

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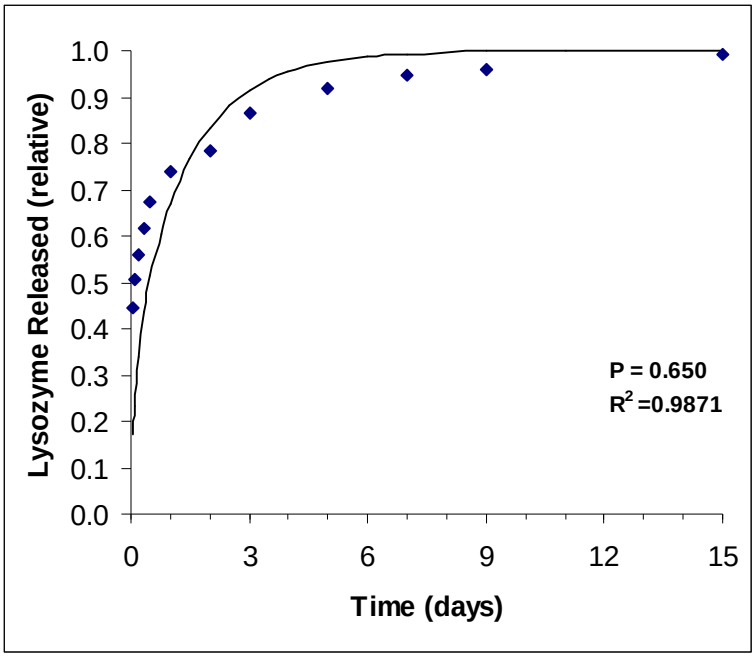

Figure 7. Fitting of the lysozyme release profile data from a representative batch with homogeneous diffusion model. P denotes the predicted permeability coefficient.

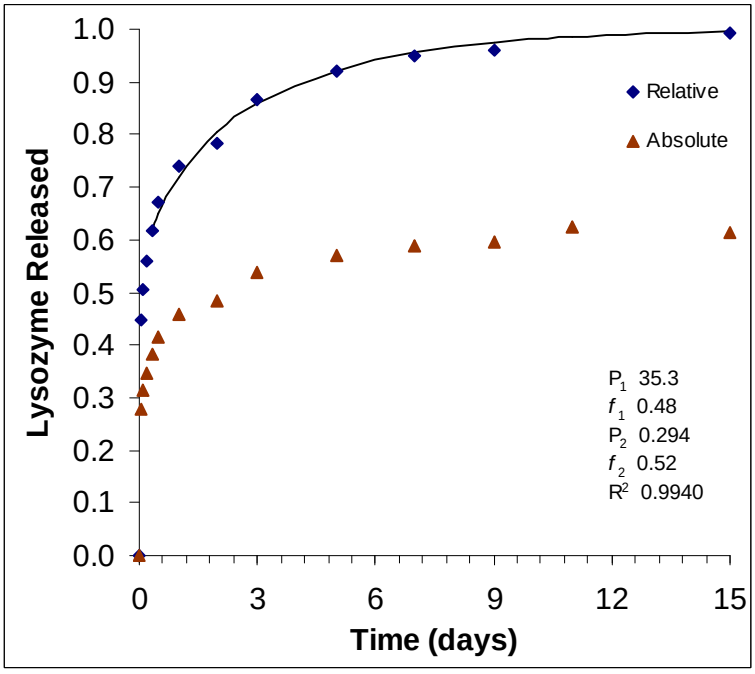

Figure 8. Lysozyme release profile from nanoparticles with DS=0.051 and lysozyme loading of 9 %. P_1 and P_2 are predicted permeability coefficients, f_1 and f_2 are the fractional contributions from each mode.

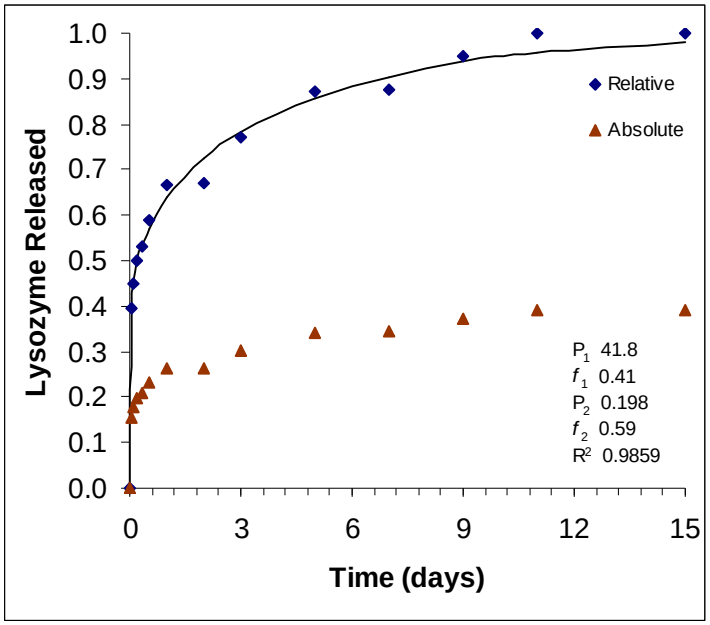

Figure 9 Lysozyme release profile from nanoparticles with DS=0.159 and lysozyme loading of 9 %. P_1 and P_2 are predicted permeability coefficients, f_1 and f_2 are the fractional contributions from each mode.

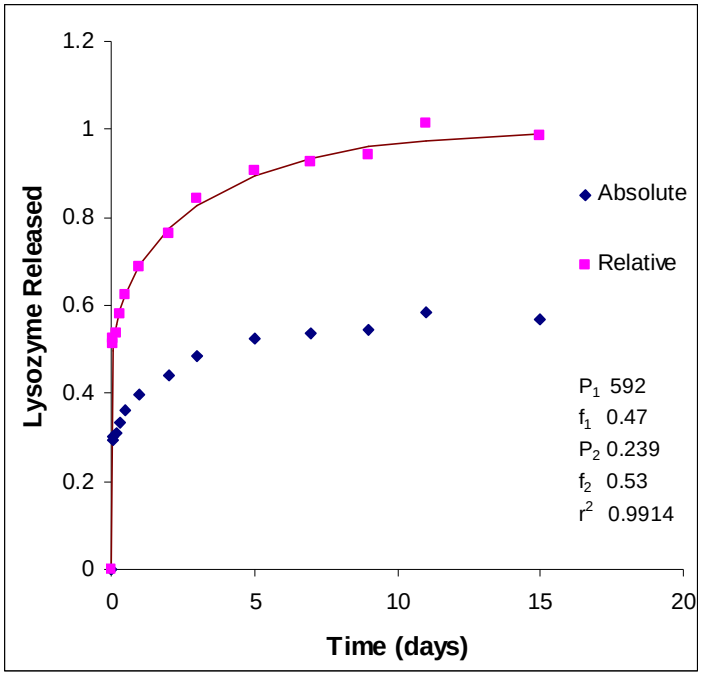

Figure 10. Lysozyme release profile from nanoparticles with DS=0.088 and lysozyme loading of 9 %. P_1 and P_2 are predicted permeability coefficients, f_1 and f_2 are the fractional contributions from each mode.

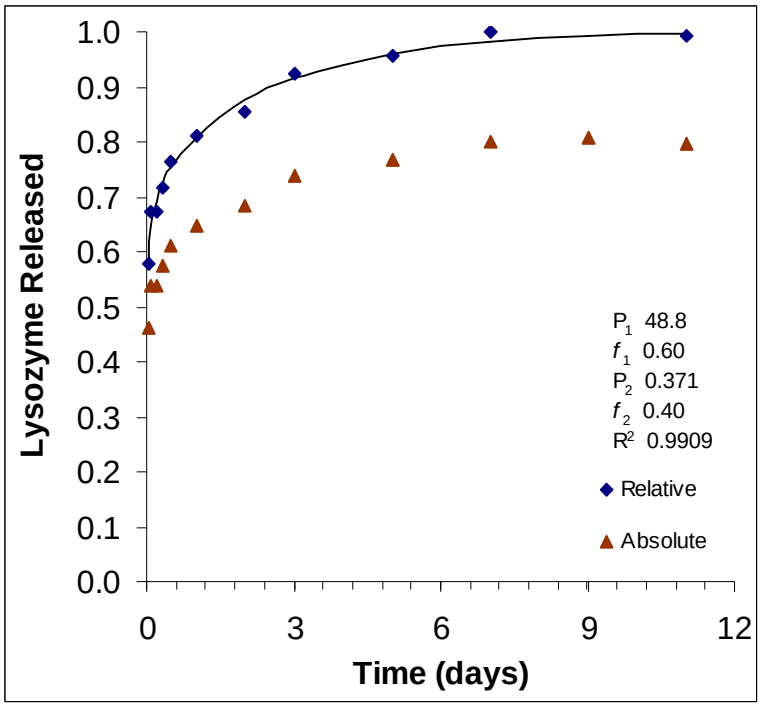

Figure 11. Lysozyme release profile from nanoparticles with DS=0.093 and lysozyme loading of 16 %. P_1 and P_2 are predicted permeability coefficients, f_1 and f_2 are the fractional contributions from each mode.

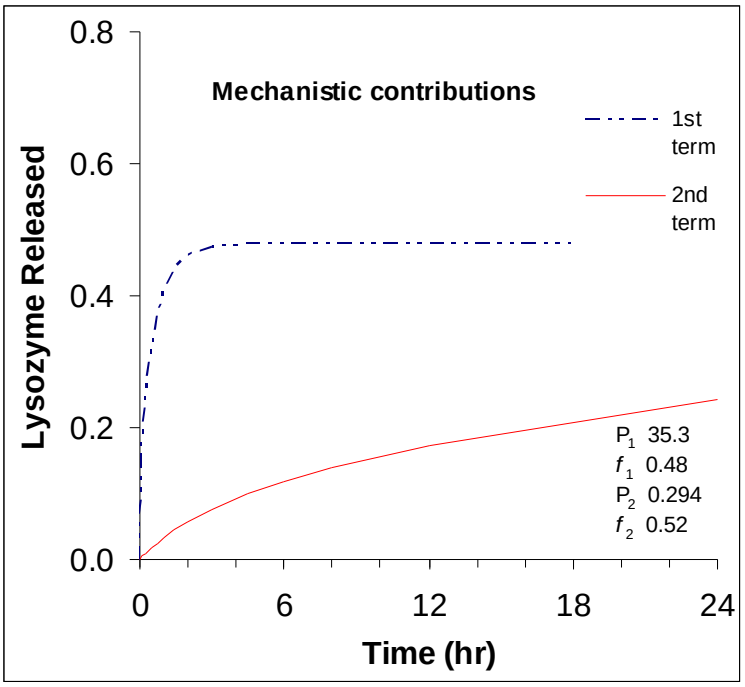

Figure 12a. Mechanistic contribution of the two diffusional modes on the lysozyme release in a representative batch (DS=0.051). A look at the scenario at a short time frame.

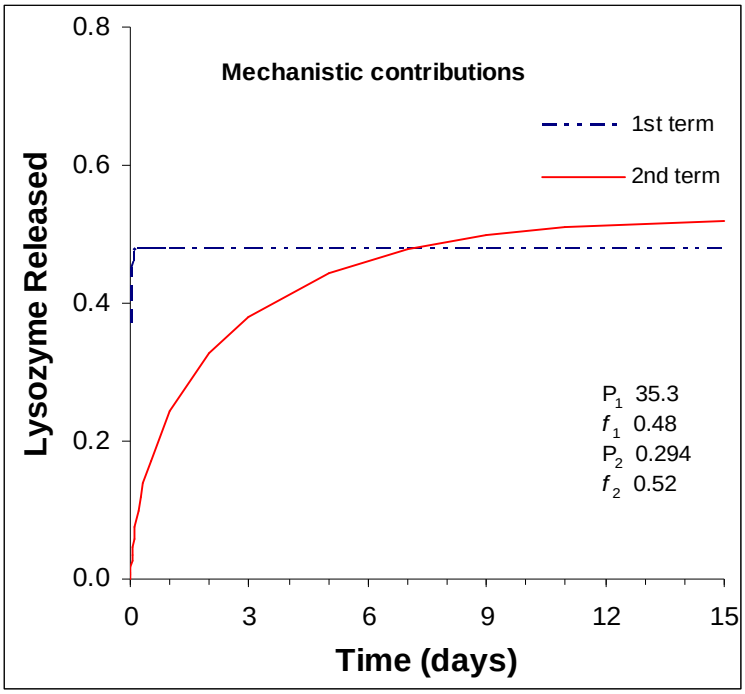

Figure 12b. Mechanistic contribution of the two diffusional modes on the lysozyme release in a representative batch (DS=0.051). A look at the scenario at a longer time frame.

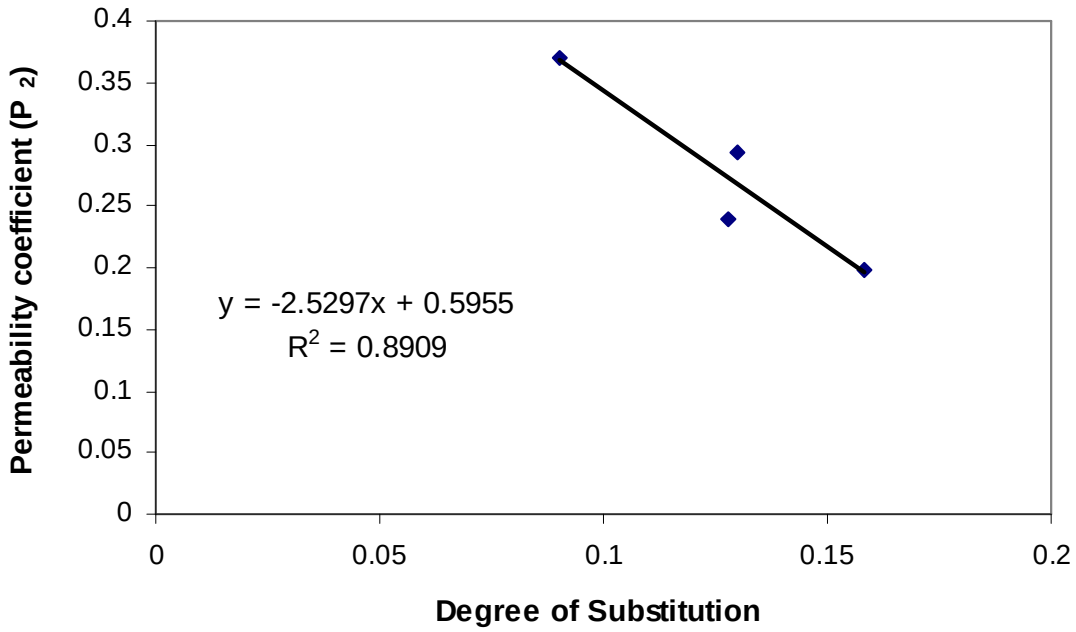

Figure 13. Relationship between the degree of substitution (number of crosslinker molecules reacted to each 100 anhydroglucose units) and permeability coefficient, P_2.

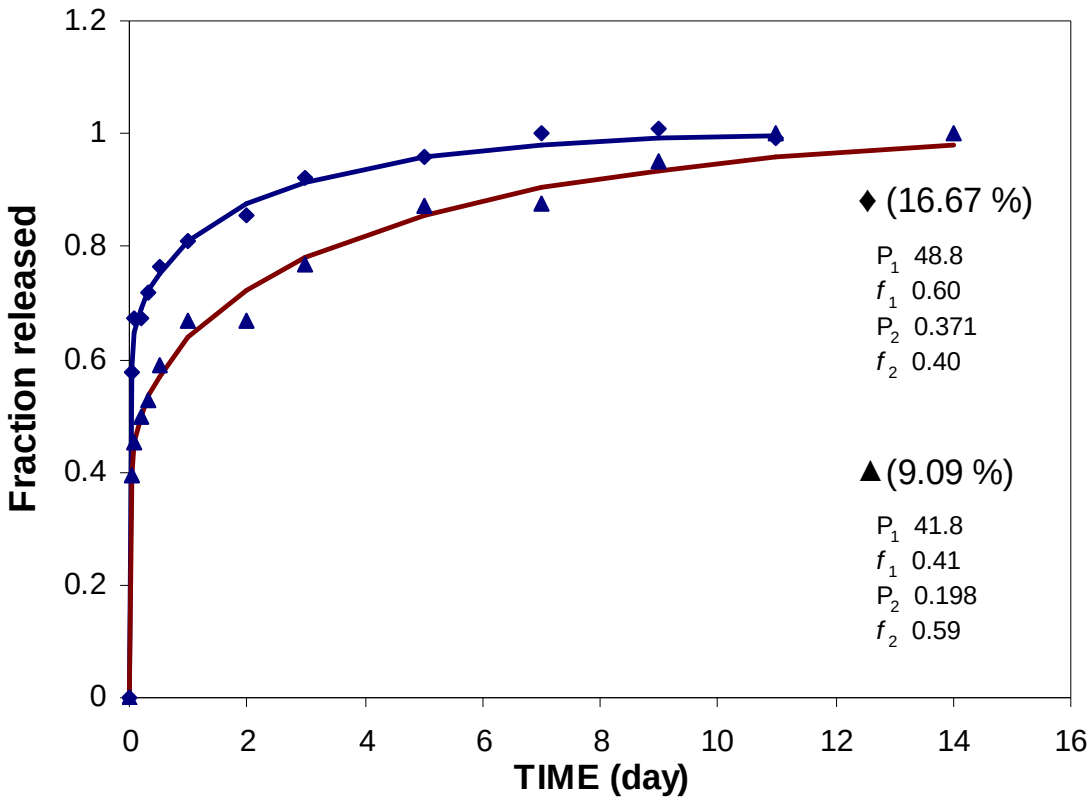

Figure 14. Comparative release profiles of lysozyme batches with different degrees of lysozyme loading. The number in the parenthesis indicates the percent loading of lysozyme compared to the dry mass of the spheres.

VI. SUMMARY AND CONCLUSION

A universal drug delivery platform that can be used beyond mere sustained delivery of bioactive molecules and for focused cellular level intervention like drug targeting and gene transfection, has been a long sought after goal of the pharmaceutical research fraternity. Due to the physiological complexity of the human body and the enormous scope of different scientific disciplines involved, the above goal is far reaching. Incremental developments in various related fields are continuously contributing to the overall progress. A number of particulate platforms composed of polymers of different physical and chemical characteristics have been developed in the last two decades and employed as sustained release injectable drug delivery systems (DDS). These DDS, while successful in sustaining the release of the incorporated bioactive compound, were found to be largely inadequate in achieving the above mentioned goal.

For cellular level intervention, injectable particulate platforms must have a long circulating half life in human blood plasma. A review of current literature reveals that there are certain conditions that have to be satisfied by the injectable particulates to be able to have a long plasma half life. The required characteristics of the particulate DDS cannot be achieved by using the traditional hydrophobic polymers like poly (lactide), poly (lactide-co-glycolide), poly (cyanoacrylate) or poly (alkyl cyanoacrylate)s.

In addition to being non toxic, biodegradable and biocompatible, the polymers used in particulate platform have to be sufficiently hydrophilic to avoid complement activation and their subsequent removal by the reticulo-endothelial system from the human body. Additionally, nanoparticles have to be monodispersed and mononucleated in an aqueous environment and of small size (< 250 nm) to achieve a long circulating half life in blood plasma. The research presented in this dissertation has been focused on developing nanoparticles which have the above mentioned characteristics and provide sustained release of incorporated bioactive molecules. The broader issues involved with cellular level intervention need to be addressed separately.

Nanoparticles have been synthesized by crosslinking carbohydrate polymers at an emulsion interface with polyfunctional acid chloride crosslinkers. Adequate characterization of the nanoparticles requires both visualization and quantification of the crosslinking achieved. An analytical method using chemical derivatization and high pressure liquid chromatographic techniques has been developed to quantify the degree of substitution achieved with the crosslinkers. This method showed adequate sensitivity and selectivity for all the crosslinkers investigated. Further, the repeatability, accuracy and precision achieved were satisfactory.

The appropriate properties of the carbohydrate polymers to be used in drug delivery platforms are biodegradability, biocompatibility, high degree of solubility in aqueous medium, minimum retrogradation and high reactivity towards the crosslinkers. All common carbohydrate polymers including cellulose, starch, starch derivatives and dextrans are hydrophilic and can be potentially used to produce long circulating nanoparticles. But only starch, starch derivatives and dextrans can be hydrolyzed by the

amylases present in the body (biodegradable) and therefore are useful for synthesizing particulate injectable drug delivery platforms.

Results of this work show that nanoparticles can be prepared from a broad spectrum of starches and starch derivatives using an interfacial crosslinking reaction. Larger polymers and polymer blends like amylopectin (waxy maize starch), corn starch, hydroxyethyl starch from corn, hydroxypropyl starch from tapioka suffer from two major disadvantages for being used for this purpose; retrogradation and low aqueous solubility. They all show retrogradation in varying degrees. Retrogradation involves changes in three dimensional configuration of the polymer and is thus expected to affect the reaction. Since the retrogradation process cannot be controlled, there is a possibility of product variability due to different states of retrogradation of starch polymers at the time of reaction. Starches also have low water solubility. Results show that the degree of substitution by crosslinkers is directly proportional to the concentration of the starch polymer in the aqueous phase. High polymer load in the dispersed phase has also been shown to improve the encapsulation efficiency of the incorporated bioactive molecules both for small molecules and large proteins.

Lower molecular weight derivatives of starch like maltodextrins have more suitable properties. They produce true solutions in water at a high concentration of 45-50 % (w/v) and do not retrograde. As a result, a relatively high degree of substitution with the crosslinkers can be achieved. The encapsulation efficiency of the incorporated bioactive molecule is improved. The possibility of product variability due to retrogradation is also eliminated. Results show that of the two maltodextrins investigated, maltodextrin from

waxy maize starch (amylopectin) is better than maltodextrin from corn starch in terms of degrees of substitution and also sustaining the release of incorporated molecules.

The surfactant is another important material component of the drug delivery system (DDS). Of the different surfactant and surfactant combinations investigated, Span 40® was found to the most stable water-in-oil emulsion with cyclohexane and chloroform. Span 40® is a nonionic surfactant that is non toxic and permitted for pharmaceutical use. When aliphatic polyfunctional crosslinkers like oxaloyl chloride or sebacoyl chloride were used, they reacted with the surfactant molecules bringing about phase separation within minutes.

Structural requirements were identified for emulsion polymerization systems. The presence of a bulky group like aromatic or tertiary dialkyl is required in between the acid chloride functionalities of the crosslinkers to prevent their reaction with Span 40®, probably by steric hindrance. Diethyl malonyl chloride (DEMC), terephthaloyl chloride (TC) and trimesoyl chloride (TMC) are three such crosslinkers which have been investigated. The first one DEMC proved to be a poor choice for this purpose because of its poor reactivity over a wide pH range of 3-11. Only at pH 10 did DEMC show any appreciable reactivity.

Both terephthaloyl chloride and trimesoyl chloride have been found to have adequate reactivity. Nanoparticles could be successfully formed with these crosslinkers with a wide variety of starch and starch derivatives. A review of available literature shows that both terephthalic and trimesic acids are found in the drinking water supply in the U.S.A. as disinfectant byproducts. A mechanism based structure-activity relationship analysis was carried out to establish the carcinogenic potential of more than 200 drinking water

disinfectant byproducts (Woo, Y. T., 2002) including terephthalic and trimesic acids. The study concluded that both terephthalic and trimesic acids are unlikely to be carcinogenic. The biocompatibility of terephthalic acid containing copolymers like poly (ethyleneglycol terephthalate)/poly (butylene terephthalate) (PEGT/PBT) is well established both *in vivo* and *in vitro* (Radersma, R. D., 2002; Bezemer, J. M., 2000). PEGT/PBT also obtained market clearance from the Food and Drug Administration in the form of a degradable cement restrictor (Radersma, R. D., 2004). Less toxicity concerns are expected from the very water soluble trimesic acid.

Results show that between TC and TMC, the later is a more appropriate choice of crosslinker for two reasons. Comparatively higher degrees of substitution can be achieved using TMC as a crosslinker and trimesic acid is more hydrophilic and highly soluble in water. Nanoparticles prepared from TC were more difficult to disperse in aqueous suspension, probably due to their relatively non polar nature of the surface and the resulting high surface tension. The optimum choice of the polymer, surfactant and crosslinker were thus found to be maltodextrin from amylopectin, Span 40® and TMC, respectively.

One significant achievement in the course of this research is that nanoparticles could be prepared with properties appropriate for long circulating half life. The prepared nanoparticles have hydrophilic surfaces (especially when prepared with TMC) and the aqueous suspensions could be visualized as discrete, monodispersed and mononucleated particles. Traditional particulate drug delivery platforms prepared from hydrophobic polymers are reported to form aggregates in an aqueous environment. The aggregates

can potentially negate the advantages of small size of the particles and can lead to thrombosis in the blood vessels and their phagocytotic removal from circulation.

The results of the investigation indicate that the resistance offered by the crosslinked membrane to the diffusional release of small molecules like tartrazine is modest. Differences could be detected among the release profiles of tartrazine from nanoparticles prepared with different polymer/crosslinker combinations. But, overall it can be concluded that achieving clinically significant sustained release of small molecules from these nanoparticles is difficult and rather unlikely. The mesh size of the crosslinked membrane of the nanoparticles is probably too large to control and/or sustain the release of small molecules significantly.

The release of larger molecules like lysozyme, on the other hand, can be controlled by the mesh size of the crosslinked membrane. Data of lysozyme release from mathematical modeling by spherical diffusion indicate two independent modes of release. In the first mode, the release is very fast, the estimated permeability coefficients are high and the release is complete within 2-3 hr. The second mode shows a much more sustained release of lysozyme over a period of weeks. The estimated permeability coefficients are much lower. A negative correlation between the degree of substitution and the permeability coefficient could be established. These results prove that the second mode of diffusional release of macromolecules incorporated in the nanoparticles can be controlled. The developed novel nanoparticular drug delivery system is thus, potentially useful for sustained release applications of macromolecules.

The present research represents a significant development towards achieving long circulating drug delivery system that can be used for sustained release applications as

well as in future efforts towards targeting. But some questions remain unanswered. Further investigations are needed to identify the cause of the fast mode of lysozyme release. The behavior of the nanoparticles in biological systems needs to be established. Surface properties of the nanoparticles must be investigated in detail; quantification of surface polarity, opsonization of the nanoparticles in circulation, their plasma half life, phagocytotic removal by macrophages and the endocytotic uptake by non-phagocytotic cells are issues of extreme interest. The full potential of the developed novel carbohydrate nanoparticles can be assessed when all the above mentioned issues are fully understood.

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