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1.1 INTRODUCTION TO NANOBIO TECHNOLOGY

Nanobiotechnology is fast emerging as one of the most productive and awe-inspiring of human pursuits (Gazit E., 2007). It has proved to be an excellent format that leads to innovative knowledge. In terms of scientific discoveries and education, it promises a sustainable environment, healthier and prosperous lives. The discipline indicates the merger of tools, ideas and materials of nanoscience with biology (Nussinov et al., 2006). Nanotechnology based ideas such as nanodevices, nanomaterials and nanoscale phenomena can be enriched through nanobiology. (Blow N., 2008). The most important objectives of the discipline involve the application of nanotools to relevant medical/biological problems. The nanotools include nanomaterials based drug delivery systems, sensors for disease diagnosis, nanophotonics based applications for studying and manipulating molecular processes in living cells and imaging of native biomolecules, biological membranes & tissues (Abu-Salah et al., 2010; Balasubramanian K., 2010; Kroll A., 2012; Leary et al., 2006). Among these, polymeric nanoparticles and amphiphilic self-assembling systems are fast gaining attention as carriers for various biomedical and pharmacological applications (Faraji et al., 2009; Nair et al., 2007). In particular, they have been used as controlled-release and targeting devices and for protecting unstable or labile molecules like drugs, enzymes, catalysts etc. (Liu et al., 2009) from hostile environments. Also polymeric nanomaterials serve as matrices for binding of proteins (Yu et al., 2008). Immunodiagnosis is another domain, where nanocarriers can be used for bio-imaging and detection of biomolecules (Quach et al., 2011; Xu et al., 2011).

1.2 POLYMER MICROREACTORS

Nanomaterials based upon microspheres, microcapsules, and liposomes are routinely used as therapeutic carriers for biomedical applications. However, it still remains a challenge for the scientific world to design and formulate micro- and nanocontainers for desired biological applications. One of the most investigated topics within encapsulation technology is the use of micro & nanocontainers and particles for immunodiagnosis and for drug delivery applications.

In the past decade, interest in nanomaterials based on polymers and amphiphilic systems has increased enormously. Using self-assembly, these synthetic structures can mimic biological macromolecules. One of the major motivations
include protection of functionally active constituents, reduction in loss of nutrients, preservation of flavors, controlled release of encapsulated materials, reduction in drug dosage and delivery of therapeutic compounds to specific locations (Gouin S., 2004; Madene et al., 2006; Maji et al., 2007). In the food industry, microencapsulation is commonly used for enveloping of flavoring agents, oils, fats and enzymes. It protects them from stringent environmental conditions such as high light intensity, susceptibility towards oxidation and high moisture. It results in increased durability of the product, reduction in volatility or transferring liquid to solid for dry mixing (Anal et al., 2007; Champagne et al., 2007; Krishnan et al., 2005; Shaikh et al., 2006). Likewise enzymes can be confined in microcapsules to accelerate ripening of cheese and improvement in its flavor (Hsieh et al., 2009). In agriculture, waxes asphalt and polymers such as polyurethanes are used to encapsulate water-soluble fertilizers in order to avoid higher concentration of fertilizer. Similarly, microencapsulation can also be used for controlled & site directed release of therapeutic molecules in pharmaceutical industry, (Dai et al., 2005; Freitas et al., 2005). Likewise, polymer based microencapsulation can also provide protection to a living cell from the immune system of the host organism (Murua et al., 2008; Orive et al., 2004). It simultaneously allows the diffusion of nutrients, oxygen and waste, which can reduce high dosage of immuno-suppressants. This technique can be used to treat numerous medical ailments which include diabetes, cancer, and neurological disorders (Barsoum et al., 2003; Maji et al., 2007; Morishita et al., 2008; Spuch et al., 2010; Wilson et al., 2009).

It is hard to attain complete impermeability to the encapsulated materials owing to the small size and reduced thickness of the microcapsules wall. However, the longevity of core materials can be enhanced by selecting suitable wall components and appropriate size of the capsule. The core material can be released by opening the wall of the capsule and this can be achieved be both mechanical as well as chemical means. Mechanical methods involve shearing, munching or crushing from outside or inside by heating the core material above the boiling point. Chemical methods include dissolution, melting or burning which result in disruption of the wall. In order to prevent leakage of encapsulated material, the polymer should not be solubilized in the similar solvent as that of the core material.

Among various types of carrier species, microcapsules based upon biodegradable polymers have been extensively studied for drug delivery applications
behind this extensively examined theme is the need to find novel materials for various biomedical applications such as disease diagnosis, controlled & targeted drug delivery (Chilkoti et al., 2006; Faraji et al., 2009; Gross et al., 2002; Liu et al., 2009). Extensive efforts by researchers have led to development of two main classes of polymer based vectors: nanospheres and nanocapsules. Nanospheres are characterized by presence of numerous porous in the wall (Fig. 1.1a) while nanocapsules comprise of polymer shell surrounding a void (Fig. 1.1b) (Benito S. M., 2006). Polymer micro/microreactors are a special class of nanocapsules which consist of a polymer shell or membrane surrounding a cavity, thus acting as encapsulating or reservoir structures (Nardin et al., 2000). These systems provide a compartmentalized volume for biochemical reactions, thus protecting unstable or labile molecules (enzymes/catalysts) from hostile environments. They can also be used for removal of pollutants and can also be directly subjected to microencapsulation of drugs, vaccines, proteins, hormones or pollutants etc. using natural or synthetic polymers (Ding et al., 2009).

![Fig 1.1: Schematic representation of difference between a nanosphere and nanocapsule.](image)

### 1.3 GENERAL CONCEPT OF ENCAPSULATION

Nature has been the most common and inspiring source for the development of encapsulation technologies. The most unique example of natural microencapsulation is a biological cell. The rigid cell wall of plants protects the inner subcellular organs from the outer undesirable environmental conditions. Likewise, the selectively permeable plasma membrane acts as a barrier and controls inward & outward movement of metabolites in the living organisms. This fundamental theme has potential applications in the domains of chemistry, pharmaceutical sciences as well as life sciences. Therefore, the term microencapsulation refers to encasing of liquids, gases or fine solid particles by aid of natural or synthetic polymers (Champagne et al., 2007; Desai et al., 2005). Microencapsulation can have numerous applications, which
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(Lendlein et al., 2002; Nair et al., 2007; Park et al., 2005). The encapsulated material in microcapsules is termed as the core/internal phase, which can be crystalline, amorphous, or emulsion/suspension of solids, (Fan et al., 2010). The enveloping matrix is called shell/coating/wall material/membrane. It can be a complex matrix, single layer or multilayer and can consist of one type or variety of materials (Li et al., 2008). A microcapsule can be permeable, semi-permeable or impermeable. The choice of shell materials depends upon various factors such as the physico-chemical properties of the core materials; the method used for synthesis of the microcapsules and the required properties of the product. The dimension of microcapsules ranges from several hundred nanometers to a few thousand micrometers. They can be synthesized in various forms such as free-flowing solid powders or as aqueous suspensions. This physical form depends on the encapsulation perspective, stability of the capsules and the encapsulated material. The subsequent release profile of the microcapsules depends upon various factors which include physico-chemical properties of the microcapsules such as average diameter, uniformity in size distribution, surface morphology, inner structure, and distribution of the encapsulated material in the core (Li et al., 2008; Park et al., 2005). The release of drugs from biodegradable polymers can be controlled by many factors, such as biodegradation kinetics of the polymer, properties of polymers and drugs, compatibility between polymers and drugs, and the shape of the microcapsules (Abraham et al., 2003; Liggins et al., 2004; Liu et al., 2004; Petrak K., 1990; Yang et al., 2000). The release profile is generally dependent upon various parameters. These include:

- Physico-chemical properties of the core material, such as diffusivity, partition coefficient & vapor pressure.
- The thickness, porosity, permeability and reactivity of wall material also affect the release pattern of the encapsulated material.
- Finally, the capsule wall should be biocompatible and biodegradable for drug delivery applications.

1.4 SCOPE OF THE THESIS

The aim of the thesis is to synthesize polymer microreactors for encapsulation of nanoparticles and drug molecules for immuno-diagnostic and drug delivery applications. Microreactors are usually fabricated by removal of the template material already coated with the material of choice. (Boyer et al., 2010; Eiden et al., 2002;
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Caruso et al., 2000). Other approaches such as emulsion polymerization (Musyanovych et al., 2007), phase separation (Pekarek et al., 1994), cross-linking of micelles (Huang et al., 1999, Li et al., 2004) and directed self-assembly (Hentze et al., 2003) have also been reported. The encapsulation procedure in all these hollow structures is driven by diffusion which is a slow phenomenon. To overcome this predicament, Im et al., (2005) reported a new method based upon instant freezing under liquid nitrogen (LN₂) followed by vacuum drying. It relies upon vacuum driven phase separation of vitrified polymer droplets which results in formation of hollow polymer microparticles. The material in question can be directly loaded through voids. Thus it becomes easier to directly encapsulate the functional material. However, the technique is not greatly understood in terms of closing of voids and also it has not been used for any type of immunodiagnostic applications.

In view of the above approach, following objectives were defined for present study:

1. Synthesis of Polystyrene (PS), Poly(lactide-co-glycolic acid; PLGA) and Poly(ε-caprolactone; PCL) microreactors.
2. Characterization of the microreactors.
3. Study of encapsulation of Quantum Dots (QDs) within microreactors.
4. Study of encapsulation of anti-cancer drug within microreactors.

In the first step commercially available latex PS microspheres (carboxylated/aminated) were examined for the formation of microreactors. Aqueous suspension of beads was swelled with toluene. Degree of swelling was governed by the volume of solvent added to the polymer suspension. The suspension of swollen beads was added drop wise into a LN₂ container. The freezed content was incubated under vacuum, maintaining the temperature below 0°C, resulting in the formation of microreactors. Electron microscopy studies affirmed substantial increase in the size of the microreactors as compared to the naive PS microspheres. When polymer and solvent were taken in 1:1 volume ratio, microreactors with a single large opening were formed. However, microreactors prepared with PS/toluene ratio of 1:2 were characterized with multiple nanometer size pores. There wasn’t much variation in the size of the microreactors compared to the native PS microspheres.

In the next step, a polymer emulsion system was used for synthesis of microreactors. The emulsion consists of two components: (a) An organic phase comprising a polymer dissolved in an organic solvent, and (b) An aqueous phase.
containing the surfactant or the stabilizer. The organic phase is then added to the aqueous phase followed by emulsification by means of mechanical stirring. The emulsion is then poured into a LN$_2$ bath and the freeze content is warmed under vacuum while maintaining the sub zero temperature. This process allows the solvent to evaporate gradually. For this PCL was chosen as standard polymer because it can be degraded under biological environment. Effects of three synthesis parameters viz. polymer concentration, stabilizer concentration and stirring speed on size and morphology of microreactors were examined. Average diameter of microreactors at 0.5, 1, 2, 5 and 7% polymer concentration was noticed as 36.89±3.93, 43.18±2.59, 55.06±2.88, 62.01±2.22 and 62.80±2.23 μm respectively. Porous structures were obtained in the range of 0.5 to 2% polymer concentration, while a big opening was observed on the surface of microreactors prepared at 2 to 7% polymer concentration. Mean size of microreactors at 1, 2, 4, 6, 8 and 10% poly vinyl alcohol (stabilizer) were 36.89±3.93, 31.7±4.84, 25.65±1.84, 9.2±3.22, 9.17±3.50 and 6.42±2.51 μm observed respectively. Bowl shape structures were noticed from 4 to 10% poly(vinyl alcohol) concentration. Likewise average diameter of microreactors at 600, 800, 1000, 1200, 1400 and 1600 rpm was 36.89±3.93, 25.64±2.11, 18.84±4.84, 16.43±2.84, 13.33±3.89 and 11.76±2.50 μm respectively.

The next approach involved synthesis of PCL and PLGA microreactors by application of strong vortex to the polymer solution mixed with the aqueous phase. For this 0.5% polymer solution was prepared in dichloromethane (DCM) and mixed with the aqueous phase containing poly styrene-alt-maleic acid (PSMA) as stabiliser. Appearance of a large void on the surface of the polymer particle affirmed the formation of microreactors.

In the next step, effect of four plasticizing solvents viz. 1,4-dioxane, toluene, DCM and chloroform was studied on closing of voids formed over microreactors. 1 mg/ml aqueous suspension of the PS microreactors was prepared and different concentrations of the plasticizers mentioned above were added. The systems were gently shaken for 1-3 hr at 37°C. It was observed that the microreactors were positively converted into microcapsules with core-shell structures in presence of 3% chloroform under 1 hr. The hollow PS particles have higher density of carboxyl/amine groups on their surface as compared to the exposed interior surface. Thus their interior surface is more hydrophobic than the exterior surface. When the hollow particles are treated with chloroform, it contributes towards minimization of surface free energy of
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The freeze-drying process has potential applications in encapsulation of nanoparticles and controlled release of therapeutic compounds. This feasibility was investigated by encapsulation of QDs in PS microreactors. Electron micrographs established the incorporation of QDs in the microreactors, thereby confirming the formation of QDs loaded microcapsules. However the photoluminescence intensity of the microcapsules decreased than the uncapped QDs. Nevertheless these fluorescent microcapsules were self-assembled on amine functionalized glass surface using Succinimidyl trans-4-(maleimidyl methyl) cyclohexane -1-carboxylate (SMCC) and thioglycolic acid (TGA). The cross-linking reaction was performed in the presence of N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). The self-assembly of the microcapsules was assessed by confocal microscopy and energy dispersive x-ray (EDX) spectroscopy. PLGA/PCL microcapsules produced by emulsification/freeze-drying can potentially be used as carriers for controlled release. The microreactors were effectively converted into DOX loaded microcapsules in presence of 3% chloroform. Drug loaded PLGA microcapsules with drug/polymer ratio 1:2 & 1:4 were referred as F1 & F2 while those of PCL with similar drug/polymer ratio were labeled as F3 & F4. Electron microscopy studies confirmed the incorporation of the drug molecules in the core of the microcapsule. The average diameter of the formulations F1, F2, F3 & F4 was found to be 123.8, 225.9, 153.8 & 216 nm with polydispersity index (PDI) of 0.158, 0.285, 0.197 & 0.454 respectively. The encapsulation efficiency measured for the formulations in similar manner was 65.56±0.126, 54.28±0.035, 61.79±0.0.092 & 56.15±0.528% respectively. Thus it showed that the amount of the drug encapsulated was strongly dependent on the drug/polymer ratio used in the experiment. In vitro release of DOX from the microcapsules was studied in phosphate buffer saline (PBS; pH 7.4) medium. The drug was released in two phases: initial phase showed a burst release of 4% of the encapsulated DOX from the microcapsules during the first 8 hr of encapsulation. It was observed that majority of the encapsulated drug was released
over a period of 15 days. The formulations F1 & F3 presented cumulative drug release value of 99.56±0.037 & 98.38±0.024% while the corresponding value determined for the formulations F2 & F4 was 95.98±0.036 & 93.24±0.045%. Thus among both the polymers, DOX encapsulated PLGA microcapsules with drug/polymer ratio 1:2 (F1 formulation) were found to be the best formulation in terms of least mean size, highest encapsulation efficiency (%) and highest cumulative (%) drug release.

Thus, polymer microreactors are highly effective tools which can be engineered for diverse nanobiotechnological applications such as disease diagnosis and drug delivery. With increase in our understanding of their synthesis & encapsulation mechanisms, it will be highly beneficial to exploit them for healthcare perspectives.