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Combinatorial Approach in the Design of Multifunctional Polymeric Nano-Delivery Systems for Cancer Therapy

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Abstract

There have been significant advances in our understanding of cancer as a disease at the molecular level and combined with improved diagnostic systems, the concept of personalized medicine has been introduced where therapy for every patient can be customized according to their disease profile. The nanotechnology approach for formulation design and the advent of drug delivery systems for small molecules and biologics has contributed to the development of personalized medicine but despite the progress, the effective management and treatment of cancer remains as a clinical challenge. The majority of drug delivery vectors that have undergone clinical trials have been discontinued prematurely due to poor therapeutic outcomes, off-target effects and non-specific toxicity owing to the components of the formulation itself. Therefore there is an urgent unmet need for a systematic approach to design drug delivery vectors that not only deliver the cargo to the desired site of action but also are highly biocompatible and non-toxic. The past decade has seen the evolution of a combinatorial approach to drug delivery, a concept that has been classically successful in novel drug discovery research. In the present review, we summarize the wet-lab and *in silico* strategies to designing libraries of biocompatible delivery materials using combinatorial chemistry and support this strategy with pre-clinical success stories in cancer therapy.

1. Introduction

Cancer as a disease is an ever-evolving medical condition that severely limits the successful outcome of therapeutic approaches primarily due to anatomical and physiological complexities as well as tumor heterogeneity. Surgical debulking followed by radiation and chemotherapy is a classical approach for containment of the disease but emergence of acquired multidrug resistance (MDR) in the majority of cancer types renders many of the frontline drugs ineffective leading to poor prognosis. Therefore, the central paradigm of cancer therapy has been modified to accommodate novel approaches that counter the predicaments of conventional therapy and exploiting the benefits of biological macromolecules (biologics) such as antibodies, plasmids and small oligonucleotides as standalone therapy or in combination with anti-cancer drugs. However, similar to chemotherapeutics, these biologics suffer from poor *in vivo* stability, short half-life, poor bioavailability, and off-target effects. The cumulative *in vivo* profile demonstrates non-specific toxicity and compromised efficacy. The development of advanced drug delivery systems has made a significant breakthrough in overcoming many of the challenges associated with the delivery of small molecules and biologics. A drug delivery vector offers considerable advantages such as improved solubility of poorly soluble drugs, protection of the payload from degradation in the body, and increased drug/payload residence time resulting in better pharmacokinetic and pharmacodynamic profiles and a subsequent improvement in efficacy and reduced non-specific toxicity[1]. Most importantly, the surface of a drug delivery system allows for ligand modifications that aid in prolonging circulation, increasing permeability and retention, as well as targeting specific types of cells or organs in the body[2]. To this end, different nanoparticle platforms have been developed for successful delivery of chemotherapeutic molecules and biologics for diagnosis, imaging and therapy of various diseases including cancer [3-6]. A typical nanodelivery vector has two important components; the material used for designing the nanoparticle that impart desired physical and biological properties and the delivery cargo that could be a chemotherapeutic drug, biological molecule, diagnostic or imaging agent. Choice, selection and manipulation of material for nanoparticle design are extremely vital criteria that will be the prime focus of this review.

The traditional approach for selecting an ideal biomaterial for drug delivery applications involves screening for certain primary desired properties including biocompatibility, toxicity, immunogenecity, biodegradability, and drug/biologics loading, protection, and release profile.

Certain physical and biological characteristics, such as particle size, surface charge, stability, targeting capability, and tissue penetration are also of prime importance in formulation design to achieve the desired performance of a delivery system for a payload of choice. This approach has proven to be effective but is also cumbersome, time consuming, expensive and an inefficient method for high throughput designing of delivery systems. In an era, where the concept of drug discovery and therapy has shifted from individual small molecule drugs to personalized therapy involving customized therapeutic regimens with a variety of different chemical and biological molecules, there is an urgent need for a modular platform for drug delivery that can accommodate the diversity of the payload. Therefore, recent efforts have focused on rationale design of a diverse variety of derivatives of platform materials to develop a library of novel materials that can be mixed and matched to develop delivery systems with specified characteristics; a concept popularly known as combinatorial design. The combinatorial approach for synthesis has been extensively explored in drug discovery but recent endeavors have proven its potential in customized material design[7]. This review will highlight different strategies adopted for developing material libraries, *in silico* approaches for the systematic analysis of material and payload properties to develop predictive capabilities for material-payload compatibility and advantages of the combinatorial approach for synthesizing materials.. We further highlight some illustrative examples where customizable drug deliver systems have been successfully applied for formulation development and pre-clinical studies of individual therapeutic molecules or combination of drugs.

2. Cancer Therapy: Systemic Delivery Challenges

2.1 Cytotoxic Chemotherapy

Chemotherapeutic toxicity can be exhibited as on-target or as off-target toxicity. On-target toxicity such as rash associated with the use of epidermal growth factor receptor inhibitors[8] or hypertension observed following the use of vascular endothelial growth factor receptor inhibitors are class effects, which are difficult to avoid by designing different active molecules without loss of activity. On the other hand, off-target toxicities are generally observed when therapeutic agents inhibit or affect other unintended targets. This occurs as the off-targets share structures or residues with the intended targets. Although these toxicities can be minimized by structural drug design to increase selectivity towards the target in many circumstances complete selectivity is not feasible or desirable. Conventional chemotherapeutic agents such as paclitaxel, etoposide, doxorubicin target

rapidly dividing cells including cancer cells and certain normal tissues. The non-specific effect on healthy tissues leads to several off-target toxic effects such as alopecia, gastrointestinal symptoms and myleosuppression. Although these traditional chemotherapeutic agents remain the treatment of choice for many malignancies, targeted cytotoxic agents (such as bevacizumab, imatinib, sorafenib) are being used for the treatment of many cancers including breast, colorectal, lung and pancreatic cancers as well as lymphomas, leukemia's and multiple myelomas [8]. These targeted therapies block the proliferation of cancer cells by interfering with specific molecules required for tumor development and growth providing treatment specificity and minimizing off-target toxicity. Although these targeted therapies show lower off-target toxicity these agents have been reported to exhibit a similar frequency and severity of toxicity as traditional cytotoxic agents with the difference being in the nature of the toxic effects [9]. Thus alternative strategies of improving tumor specific delivery while minimizing off-target toxicity are continually being investigated to provide patients with optimal cancer therapy.

2.2 Nucleic Acid Therapies

Nucleic acids are large, hydrophilic and highly negatively charged molecules which when administered as foreign agents can be recognized and degraded by the immune system. Several different types of molecules that act to inhibit gene expression by sequence-specific targeting of mRNA's have been developed as potential therapeutic agents. Short interfering RNA (siRNA) and microRNA (miRNA) are two such nucleic acid therapies (NAT) that have been pursued for cancer therapy. siRNA's are RNA fragments approximately 21-23 nucleotides long that are capable of inducing the sequence specific destruction of complementary mRNA [10]. On the other hand miRNA's are small endogenous molecules that regulate gene expression by direct binding to the 3-UTR of coding messenger genes resulting in their translational repression and/or mRNA degradation [10].

Although these NAT's can be delivered in their naked form (non-carrier based approach) or through a non-viral or viral delivery system, the non-carrier based approach has been used for the local delivery of therapeutic agents (in macular degeneration, wound healing and infectious respiratory disease). However as systemic delivery provides access to many tissues, systemic delivery of NAT's has been pursed with interest. Systemic delivery of non-carrier based NAT's has been limited due to poor ribonuclease resistance (RNase), which yields a very short half-life

(2-6 min) for these therapies [11]. Naked NAT's also do not bind strongly to plasma proteins nor do they accumulate in body tissues. On the other hand, due to their small size they are easily cleared through the kidneys (17.6 $ml.min^{-1}$) [11]. Due to their poor plasma stability, poor tissue specific accumulation and rapid clearance, non-carrier based NAT's have to be dosed in excessive quantity to reach a particular location in the body and be retained for a therapeutically optimal period.

2.3 Multidrug Resistance

Drug resistance to anticancer agents is typically attributed to impaired delivery or due to genetic/epigenetic alterations, which affects drug sensitivity. These resistance mechanisms led to the development of cross-resistance to other structurally and mechanistically unrelated drugs inducing the development of multidrug resistance (MDR). Impaired delivery of cytotoxic agents to the tumor site occurs due to poor absorption of orally administered drugs, increased drug metabolism or increased excretion of the drug which lowers the drug concentration at the target site. Apart from the pharmacokinetic effects, cancer cells have resistance mechanisms to negate the therapeutic effects of cytotoxic agents. Natural product hydrophobic drugs are substrates for innate multidrug resistance mechanisms, which results from the expression of ATP-dependent efflux pumps that have broad drug specificity. These pumps increase drug efflux, which leads to lower intracellular drug concentrations. Vinca alkaloids (vinblastine, vincristine), anthracyclines (doxorubicin, daunorubicin), RNA transcription inhibitor (actinomycin-D) and microtubule stabilizing drugs (paclitaxel) are typical examples of compounds effluxed from cells using innate multidrug resistance mechanisms [12]. Apart from increased efflux, cancer cells develop resistance by reducing drug uptake of compounds such as methotrexate, 5-fluorouracil and cisplatin transporters and carriers that typically bring nutrients into the cell. Drug resistance can also be developed by regulating endogenous detoxification systems for example DNA repair and CYP450 mixed function oxidases, which minimizes the effectiveness of the drug. Malignant transformation of cells leads to defective apoptotic pathways such as alterations in ceramide levels or changes in cell cycle machinery which activate check-points preventing the initiation of apoptosis and thus promoting resistance. As cancer cells are genetically heterogeneous, more than one mechanism plays a role in the development of resistance a phenomenon termed multifactorial multidrug resistance.

2.4 Strategies to Overcome Tumor Resistance

Several strategies have been investigated to overcome tumor resistance including combination therapy with two or more drugs, protein inhibition and gene silencing. In combination therapy, cytotoxic agents with different mechanisms of action are combined to target multiple sites in the cancer cell. However, due to the phenomenon of cross-resistance this approach often does not provide an adequate therapeutic outcome.

MDR protein inhibition is a strategy that is used for cytotoxic agents typically pumped through the P-gp efflux systems. In these situations MDR protein inhibitors such as cyclosporine, verapamil and tariquidar are often combined with cytotoxic agents to attain high intracellular drug concentration. With the development of nanoparticle-based therapy in cancer drug delivery, co-delivery of MDR protein inhibitors and cytotoxic agents can be achieved. Co-encapsulation of cyclosporine A and doxorubicin showed a two fold higher efficacy in doxorubicin resistant leukemia cells compared to using free cyclosporine A or only doxorubicin loaded particles [13]. Similarly transferrin conjugated (receptor targeted) liposomes with verapamil and doxorubicin showed faster internalization of the nanoparticles and greater accumulation of doxorubicin in doxorubicin resistant leukemia cells [14]. Thus the targeted delivery of chemosensitizers and cytotoxic agents using nanoparticles is a safe and effective approach for the treatment of cancers resistant to chemotherapy. Apart from the delivery of cytotoxic agents and chemosensitizers, nucleic acid loaded nanoparticles have been used to silence P-gp expression to increase intracellular drug concentrations. These particles have shown higher systemic stability as well as target specificity compared to free siRNA, which is unstable in serum and demonstrates poor cellular uptake.

3. Polymeric Nano-Systems for Systemic Delivery in Cancer

3.1 Targeted delivery systems

The targeting of nanoparticles can be classified into passive targeting and active targeting. Passive targeting relies on the Enhanced Permeability and Retention effect (EPR), which is characterized by enhanced accumulation of nanoparticles within tumor tissues due to the leaky tumor vasculature and impaired lymphatic drainage [15]. For passive targeting, nanocarriers have to circulate in the blood for sufficient, prolonged periods. This goal can be achieved through

optimizing stealth properties, typically by physical incorporation or chemical conjugation of polyethylene glycol (PEG) into the shell of the nanocarriers. Among various polymeric nanocarriers, polymeric micelles prepared from self-assemblies of amphiphilic PEG-based block copolymers have received great interest for tumor targeting via the EPR effect [16-18]. The dense PEG shell of the micelles prevents protein adsorption and recognition by the phagocyte system, increasing the blood circulation time of the nanocarriers. We have shown that passively-targeted PEG-modified type B gelatin-based nanoparticles are very effective for gene delivery to solid tumors [19, 20]. The extravasation of polymeric nanocarriers into tumor tissues and penetration of the nanocarriers within the tumor tissue is size-dependent. It is currently thought that nanocarriers in the 10-100 nm size range and with a surface charge either slightly positive or slightly negative are often not cleared by renal clearance and the reticuloendothelial system, enablingaccumulation at the tumor site after prolonged circulation [5]. Despite the popularity of the EPR effect in cancer research, it is argued that no more than 5% of the injected dose extravasates and accumulates in tumors[21]. The extent of nanoparticle extravasation depends heavily on a number of factors including the degree of capillary disorder, blood flow, and the rate of lymphatic drainage, which varies among tumor types [15, 22]. For example, certain tumors, such as metastatic liver, pancreatic and prostate cancer, bear intrinsically low vascular densities, in which the EPR effect strategy may not apply in the core of a large-sized tumor (*e.g.*, 1–2 cm in diameter) due to the absence of densely vascularized structures [23]. In a recent study, the Kataoka research group compared the accumulation and effectiveness of different sizes of long-circulating, drug-loaded polymeric micelles with diameters of 30, 50, 70 and 100 nm in both highly and poorly permeable tumors. All the polymer micelles penetrated highly permeable tumors in mice, but only the 30 nm micelles could penetrate poorly permeable pancreatic tumors to achieve an antitumor effect [24]. The study also showed that the penetration of the larger micelles could be enhanced by a transforming growth factor inhibitor to increase the permeability of the tumors [24]. To achieve homogenous accumulation, polymeric nanocarriers need to move deeply into the tumor tissues after extravasation. However, the transport of the nanocarriers is largely impeded by the high interstitial fluid pressure (IFP), which also hinders the nanocarrier retention in tumor tissues. In addition, tumor accumulation of drugs in tumor tissue does not always guarantee successful therapy if the drug does not reach the target site of the tumor cell such as the cell membrane, cytosol, or nucleus. For effective cancer therapy, it is critical to precisely guide nanocarriers to a specific cell type or a

specific non-cellular component in the tumor microenvironment, which could be achieved through actively targeted delivery systems.

To achieve tumor specificity, various polymeric nanocarriers employ active targeting. Active targeting exploits tumor cell characteristics, such as over-expression of specific antigens or receptors on their surfaces that are at low levels in normal tissue cells [25]. Through careful engineering of polymeric nanoparticles, various targeting ligands such as antibodies, peptides, nucleic acid aptamers, carbohydrates and small molecules can be displayed to enhance selective delivery to tumor site while decreasing the localization in the liver and spleen [26]. Binding affinity of the targeting ligands with their receptors influences the tumor penetration of the nanocarriers. For targets in which cells are readily accessible, typically the tumor vasculature, high affinity binding is preferable. So far, the targeting moieties have been used to either directly target the cancer cells; or to target the tumor vasculature endothelial cells and indirectly inhibit cancer cell growth by deprivation of the oxygen and nutrients carried by tumor vasculatures [27]. The aim of targeting cancer cells is to enhance the cellular uptake of the nanocarriers. Thus, the active targeting of cancer cells is particularly attractive for the intracellular delivery of DNA, siRNA, and protein. The enhanced cellular uptake rather than an increased tumor accumulation is responsible for the anticancer efficacy of the actively targeted nanocarriers [28]. The concentration of the surface ligand is a critical parameter that dictates the ligand targeting effect. High ligand density may increase the probability of nanoparticle interactions with cell receptors. However, the presence of increased non-PEG like-material on the surface of nanoparticles can be more detrimental than advantageous to delivery [26]. The popular receptors for cancer cell targeting include transferrin, folate, glycoprotein, and epidermal growth factor receptor (EGFR). One targeting ligand which has received great attention in cancer research during the past two decades is HER2. HER2 (Human Epidermal Growth Factor Receptor 2) is highly expressed in breast cancer, ovarian cancer, and gastric cancer [29]. The overexpression of HER2 is also associated with more aggressive tumor phenotypes and poor prognosis, thus making it an attractive target for cancer therapy. The succesful development of trastuzumab (Herceptin^{TM}), an anti-HER2 antibody, has had a major impact on the treatment of breast cancer [30]. Our laboratory has developed EGFR-targeted polymeric nanoparticles using an EGFR targeting peptide conjugated with gelatin nanoparticles or with a synthesized EGFR peptide/PEG construct incoorporated in polymer blend nanoparticles for treatment of pancreatic cancer and multidrug resistent cancer [31, 32]. Herceptin-conjugated

nanoparticles of D-alpha-tocopheryl-co-poly(ethylene glycol) 1000 succinate (TPGS)-cisplatin prodrug and herceptin-decorated poly(D,L-lactide-co-glycolide) have also been developed for the effective treatment of breast cancer [33, 34].

For targeting tumor endothelial cells, there is no need for nanocarrier extravasation to arrive to their target site and receptor binding is directly possible after intravenous injection [28]. The ligand-targeted nanocarriers bind to and kill angiogenic blood vessles inducing tumor cell death due to the lack of oxygen and nutrients. The main receptors of the tumoral endothelium include the receptors of vascular endothelia growth factors (VEGF), the $\alpha_v\beta_3$ integrin, vascular cell adhesion molecule-1 (VCAM-1), and the matrix metalloproteinase (MMPs). Among targeting delivery systems, $\alpha_v\beta_5$ or $\alpha_v\beta_3$ integrin targeted nanocarriers could be considered as double targeting systems because $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrin are over-expressed in both tumor cells and angiogenic endothelial cells [35]. Despite the ability to enhance cellular uptake and tumor retention, active targeting strategies can result in a high accumulation of the nanocarriers in non-target cells due to basal expression of antigens, carbohydrates, and receptors, thus toxic off-target effects can occur. An alternative approach to localize therapeutic agents inside tumor cells is the use of stimuli-responsive nanocarriers that release a therapeutic payload in response to a microenvironment trigger.

3.2. Stimuli-responsive polymeric systems

Stimuli-responsive polymeric systems maintain stealth function during circulation, upon arrival at the tumor site, drug release is triggered by a unique property of the tumor micro environment such as a change in pH, redox or enzyme gradient. Tumor microenvironment is distinctly different from normal tissue environment, which imparts key advantages to tumor by promoting growth, progression and aggressiveness. However, this provides unique opportunity to exploit these characteristic features for developing vectors that could deliver the cargo in response to the internal stimuli specific to the tumors. We briefly summarize some of the popular stimuli-responsive approaches that have been undertaken but the readers are encouraged to read detailed accounts on this aspect of research in the published literature.[36, 37]

Interstitial fluid in tumors is known to have a lower pH (6.5-7.2) than that in normal tissue (~7.4). This is mainly a consequence of irregular angiogenesis in fast-growing tumors, which causes a rapid deficit of tumor nutrition and oxygen, and a subsequent increase in glycolytic

metabolism resulting in the over-production of lactic acid in the tumor interstitium. The insufficient blood supply and poor lymphatic drainage, characteristics of most tumors, contributes to the acidity of the tumor microenvironment [38]. After cellular uptake by endocytosis, nanocarriers face very well defined compartments with strongly differential pH values. The early endosome has a pH of about 5–6 while the late lysosome, which is the most acidic compartment, has a pH around 4–5 [39]. This phenomenon has been employed to design numerous pH-responsive polymeric systems for the delivery of anticancer drugs to tumors. The pH-responsive systems are stable at physiological pH of 7.4, but destabilize to release the drugs at lower pH environments in the extracellular matrix of solid tumors and in endosomes and lysosomes. One representative example could be the zwitterionic polymer based surface charge switchable nanoparticles that overcome the weak nanoparticle-cell membrane interactions [40]. In this system, an extracellular pH-sensitive 2,3-dimethymaleic anhydride (DMA) was introduced as the anionic part of the zwitterionic polymer PCL-*b*-P(AEP-*g*-TMA/DMA). The nanoparticles show a nearly neutral surface charge in blood circulation so as to avoid rapid recognition by the immune system and exhibit delayed blood clearance time, which can increase accumulation in tumor tissue through the EPR effect. Once accumulated in tumors, they can respond to the extracellular pH and remove the negative DMA residues from the nanoparticle, and convert to a positively charged form, enhancing the NP–cell membrane interactions and facilitating tumoral cell uptake (**Figure 1**).

Glutathione (GSH) is the most abundant reducing agent in living cells. It has been established that extracellular space is oxidative while the intracellular is reductive, which is strongly related to the difference in the intracellular GSH concentration (~2-10 mM) and GSH concentration in blood and extracellular matrix (~2-10 μM) [41]. Moreover, tumor tissues has about 7-fold higher GSH concentration than that of normal tissue [42], and is often elevated in multidrug resistance cancer cells [43]. This significant variation in GSH concentration in the tumor microenvironment has been exploited to design redox-responsive polymeric nanocarriers for intracellular delivery, especially gene delivery. This has been achieved by reductively degradable micelles from self-assembled amphiphilic copolymers containing disulfide links in the repeated units of the hydrophobic backbones or bearing a single disulfide bond at the connection of the two polymer blocks [44-46]. Other approaches use GSH-sensitive crosslinking agents incorporated either in the shell or in the core of the micelles leading to rapid disassembly of the micelles followed by

intracellular release of therapeutic agents [47, 48]. Polymers ranging from chitosan to poly (ethylene imine) have been studied for redox-responsive systems through disulfide linkages.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases that are essential for angiogenesis, tumor invasion and metastasis. These enzymes are abundant in the tumor extracellular matrix and are often upregulated in invasive tumors due to the increased need for extracellular matrix degradation [49]. MMP-responsive nanocarriers can be achieved by incorporation of enzyme-cleavable peptides in the structure of nanocarriers.

3.3 Need for modular platform

Despite extensive research efforts to develop new nanoparticles for cancer therapy, currently drug delivery systems have only achieved modest therapeutic benefits [50]. Furthermore, there is an increasing need for the combination of diagnosis and therapy. Thus, the development of suitable nanotechnology platforms to improve drug delivery to tumor tissue is still a pressing need. The modular design strategy relies on the development of integrated methods for the preparation of a single nanocarrier which can address following challenges: (i) biocompatibility and biodegradability to achieve optimal benefit/risk relation; (ii) controlled size in the range of 10-100 nm, optimal stealth properties to escape immune recognition and enable the EPR effect through prolonged blood circulation; (iii) high targeting specificity for improved tumor retention and cellular uptake; (iv) controlled release mechanisms through stimuli responsiveness to trigger release of drugs or genes inside tumor cells; (v) multimodal imaging functionality for monitoring biodistribution. However, combining imaging and other functionalities in one nanoparticle can be challenging since a limited number of attachment sites are available on the nanocarrier surface making it difficult to couple several functional groups in sufficient concentration. Furthermore, some groups may interact to sterically shield or alter each other's activity when in close proximity. In addition, multiple functional moieties on a nanoparticle may also reduce colloidal stability or adversely affect the *in vivo* pharmacokinetics [51]. Therefore, it is necessary to design new nanoparticles of higher capacity and functionality. A common approach to incorporate multiple functionalities onto a single nanoparticle is the use of core/shell architecture with polymeric nanoparticles. In another approach, Schneider et al. reported a highly versatile nanoparticle-based core/shell drug delivery system prepared by electrostatic and covalent layer-by-layer assembly strategy using a gold nanoparticle core. The multifunctional shells are constructed using a single

assembly process in which various different functionalities are incorporated in a modular fashion (**Figure 2**) [52].

4. Combinatorial Design of Polymeric Nano-Systems

4.1 Concept of Combinatorial Design and Formulation Customization

The rationale behind the application of the combinatorial approach for designing novel delivery strategies has evolved from the central paradigm for high-throughput drug discovery, where the experimental and computational methods are utilized to select positive "*hits*" of drug candidates from a library of compounds [53]. This approach has been extensively used in small molecule drug development and had been further extended to the discovery of recombinant proteins, peptides and antibody candidates [54]. In perspective to drug delivery vector design, combinatorial approach integrates the ideal characteristics of materials suitable for biomedical applications to their structure-property relationship. Central to this idea is the concept of developing a modular and customizable platform that can be tailored at will to deliver a variety of payloads including small molecules, nucleic acids and proteins. To this end, polymeric scaffolds have been the most popularly used since they offer multiples advantages. Polymeric drug delivery systems have been extensively studied in the past with an abundance of literature that offers chemical synthesis approaches that are well understood. Advances in chemical analysis methods further aid in the estimation of the precise degree of modification to develop a robust and reproducible synthesis scheme. Most importantly, their biocompatibility, biodegradability and clearance mechanisms from the body has been well established, which allows for selection of *Generally Regarded as Safe* (GRAS) starting materials to develop delivery systems. These properties are key to develop clinically translatable delivery systems. Polymeric systems are also a popular choice for such applications since the repeat units of the monomers provide abundant functional groups that can be exploited for a precise degree of chemical conjugation/modification to attain a product with desired properties. Advances in high-throughput screening and combinatorial methods for drug discovery can be easily extended to monomers and polymer synthesis that would lead to the fast-paced development of building blocks that can be blended in optimal proportions to develop novel delivery systems.

The first introduction of parallel synthesis and combinatorial design of polymeric materials was reported from Dr. Langer's laboratory where 7 different diacrylate monomers were modified

with 20 different amine monomers to give a library of 140 poly (β-amino ester) (PBAE) that were further tested for their aqueous solubility and subsequent DNA complexing capability [55]. The library size was large enough to give sufficient versatility in structurally unique polymers but small enough to avoid the use of an automated screening process. This combinatorial approach was further developed and improved by the same group to extend the repertoire of the library and allow for synthesis and screening for the product for nucleic acid binding without a need for additional purification [56]. They further demonstrated that combinatorial design could be successfully extended to generate a library of lipid-like materials called *"lipidoids"* using a similar synthetic approach based on alkyl acrylate (or acrylamides) to primary or secondary amines. A library of more than 1200 structurally unique lipidoids was successfully synthesized and screened for transfection efficiency [57].

These examples of combinatorial synthesis and design of materials demonstrate the strength and versatility of the approach and the potential it offers in generating libraries of derivatives that can be explored for delivery. However, the majority of these approaches are based on conjugation of electropositive amines on to the polymer backbone to facilitate subsequent nucleic acid complexation and encapsulation. There is a deficit of studies demonstrating the application of these materials in the systematic design of delivery systems for small drug molecules, primarily due to the high variability in properties of these molecules. A methodical approach towards material selection for small molecule encapsulation and delivery should consider not only the properties of the polymer but also the candidate drug. Solubility, charge and interaction with the host polymer of the delivery system are some of the key properties that define the encapsulation efficiency of a drug and its release profile. Such multi-parametric predictions are hard to comprehend without the use of bioinformatics tools that are now extensively applied in understanding drug-material interactions, its effect on the performance of the delivery system and to some extent, predicting the material properties that would be ideal for a given drug candidate. The following section will highlight some of the modeling approaches and *in silico* validations that have been explored in a quest to discover novel delivery systems and improve the design of existing systems.

4.2 Modeling and In Silico Validation

Pharmaceutical science relies on modeling of drug delivery systems to help conceptualize, understand and predict the efficacy of the system. These modeling strategies are rich and diverse, and they include not only *in vitro* and *in vivo* but also theoretical or *in silico* approaches. In the last couple of decades mathematical and computational cancer modeling (*in silico*) has gained momentum. In 1961, the father of mathematical modeling for drug delivery systems, Professor Takeru Higuchi published his famous equation (eq. 1) allowing for a surprisingly simple description of drug release from an ointment base exhibiting a considerable initial excess of non-dissolved drug within an inert matrix with film geometry (**Figure 3**) [58, 59].

$$M_t/A = \sqrt{D \cdot (2c_0 - c_s) \cdot c_s \cdot t} \quad (1)$$

Here, M_t is the cumulative absolute amount of drug released at time t, A is the surface area of the film exposed to the release medium, D is the drug diffusivity in the carrier material, and c_0 and c_s represent the initial drug concentration and the solubility of the drug in the carrier material, respectively. An important advantage of this equation is its simplicity.

This was the beginning of the quantitative treatment of drug release from pharmaceutical dosage forms. After this, numerous models have been proposed, including empirical/semi-empirical as well as mechanistic,realistic models. Empirical/semi-empirical models are purely mathematical and do not consider real physical, chemical or biological phenomenon [60-63]. These models give no or very limited insight into the underlying drug release mechanisms. Moreover, these models usually are less accurate than mechanistic models. These type of theories might, for instance, be useful if different types of drug release profiles are to be compared using a specific parameter (e.g., an apparent release rate constant for experimental design analysis). But great caution must be paid if mechanistic conclusions are drawn or quantitative predictions are made. In contrast, mechanistic mathematical theories are based on real phenomena, such as diffusion, dissolution, swelling, erosion, precipitation and/or degradation [64-72]. These type of models allow for the determination of system-specific parameters that can offer deeper insight into the underlying drug release mechanisms.

4.2.1 Mechanistic models:

Diffusional mass transport (DMT) is of utmost importance in drug delivery systems as it represents the way a drug is released by the device. Fick's law of diffusion is used to quantify DMT [73]. Fick's second law of diffusion is as follows,

$$\frac{\partial c}{\partial t} = D \cdot \left(\frac{\partial^2 c}{\partial x^2} + \frac{\partial^2 c}{\partial y^2} + \frac{\partial^2 c}{\partial z^2}\right) \qquad (2)$$

where c is the concentration of the diffusing species; t denotes time, D is the diffusion coefficient and x, y and z are the three spatial (Cartesian) coordinates.

Here, it is to be noted that time- and position-dependent diffusion coefficient (D) might include matrix erosion, polymer swelling and/or degradation [74-76]. These scenarios lend Fick's second law (equation 2) unsolvable requiring a different set of models for these scenarios. Appropriate selection of such a model is largely determined by the following properties of the system i) physical placement of drug in the device, ii) initial concentration of drug, iii) geometry of delivery system.

4.2.1.1 Reservoir systems with a non-constant activity source: In these cases, the drug is physically completely separated from the release rate controlling material, which forms a barrier membrane surrounding the drug depot. Moreover, the initial drug concentration is below drug solubility and authors have proposed different models for these cases [77, 78].

4.2.1.2 Reservoir systems with a constant activity source: In these cases, the drug is also physically completely separated from the rate controlling barrier membrane, but the initial drug concentration is above drug solubility. So, upon water penetration into the device not all the drug is dissolved. Thus, a saturated drug solution is rapidly created in the core and released drug molecules are rapidly replaced by the (partial) dissolution of remaining drug excess. Consequently, the drug concentration at the inner membrane's surface remains constant (as long as drug excess is present). In these scenarios the dissolution rate is determined using the Noyes-Whitney equation [79].

4.2.1.3 Monolithic dispersions: If the drug is homogeneously distributed within the delivery system at an initial concentration that exceeds drug solubility, this type of device is called a monolithic dispersion. Upon contact with aqueous body fluids, water penetrates into the system and only partially dissolves the drug. Thus, dissolved and non-dissolved drug co-exist within the matrix during drug release. Importantly, only dissolved drug is available for diffusion. Hugachi's equation (equation 1) models this scenario with film geometry of a drug delivery system. Roseman *et. al.* modeled drug dispersion in this scenario with sphere and cylinder geometry [80, 81]. Paul has extended Higuchi's model to incorporate additional complexity to the system [82].

4.2.2 Empirical models:

4.2.2.1 Peppas Equation: Peppas equation[60] models drug release as a power law,

$$M_t/M_\infty = k \cdot t^n$$

Here, M_t and M_∞ are the absolute cumulative amount of drug released at time t and infinity, respectively. k is a constant incorporating structural and geometric characteristics of the system, and n is the release exponent, which might be indicative of the mechanism of drug release. It is to be noted that the classical Higuchi equation (Eq. 1) is a special case of the Peppas equation where the release exponent is 0.5. Thus, a release exponent of 0.5 can serve as an indication for diffusion controlled drug release, but only if all assumptions these particular solutions are based on are fulfilled, in example slab geometry with negligible edge effects, time- and position-independent diffusion coefficients in a non-swellable and insoluble matrix former.

4.2.2.2 Data Mining: Lipinski has derived rules such as the "rule of five" using data mining techniques for optimal bio-availability and absorption of drugs [63]. Wu *et. al.* have proposed a drug disposition classification system (BDDCS) and provided over 160 molecules as examples of four classes [83]. Researchers have used an artificial neural network (ANN) to model drug delivery system [61, 62]. An ANN consists of one input layer, one output layer and one or more hidden intermediate layers. Each layer is composed of several units, called "neurons". The input layer encompasses input values of causal factors, e.g. the drug loading, compression force etc. The output layer can, for instance, consist of constants describing the drug release profile. The above-mentioned ANN is first trained with a set of experimental results (consisting of input and output values *in vivo* or *in vitro*). Once the system is "trained", it can be used to make quantitative predictions for the output values based on new input values.

4.3 Synthesis Approaches

The methods adopted for synthesizing materials by combinatorial approaches for high-throughput screening should fulfill some important criterion for their successful implementation. Firstly, the synthesis scheme should be a single step, straightforward process that can result in a product with high consistency and should be amenable to scale-up. Researchers have focused on methods that can avoid complicated reaction conditions, need of catalysts and significantly higher volumes of solvents. Secondly, the choice of reaction should ideally avoid the formation of by-products or intermediate products that would need additional purification steps before the material could be utilized for characterization and delivery applications. Most importantly, an ideal

synthesis method should be able to use the entire raw material and convert it into product to avoid waste of material especially when the polymeric backbone is expensive. To this end, various approaches have been used that meet the described parameters and have been successfully used for the generation of polymeric materials.

4.3.1 Amine Addition: The contribution from Dr. Langer's work has popularized this synthesis approach where primary or bis(secondary) amines are added to a poly(β-amino ester) to yield cationic polymer products [55-57]. This method has three distinct advantages: the raw materials are inexpensive, the reaction proceeds to completion in a single step and most importantly, it does not yield any by-products, thereby avoiding a need for product purification. Importantly, the reaction conditions can be tailored to accommodate polymers with varying molecular weights ranging from 2000 to 50000 Da, demonstrating the versatility and adaptability of the synthesis methodology [55]. Such flexibility in the synthesis approach allows rigorous study of the structure-property relationship of materials and allows optimization to achieve better materials. Anderson *et al.* studied parameters such as molecular weight, particle size and surface charge after nucleic acid complexation, optimal polymer/nucleic acid ratio and its effect on transfection efficiency [84].

4.3.2 Amidation: Amidation is the process of the formation of an amide bond using a functional carboxylic and amine groups and largely relies on a carbodiimide coupling reaction in the presence N-hydroxysuccinimide (NHS). Carbodiimide coupling is particularly attractive since it can be carried out using water as the solvent and the reaction does not require special conditions such as temperature, pH or a catalyst for completion. 1-ethyl-3-[3-(dimethylamino)-propyl]-carbodiimide (EDC) has been a preferred choice due to its high water solubility. However, this reaction suffers from poor yield of the product and usually excess of EDC and NHS are used due to propensity of these compounds to undergo hydrolysis. Replacing water with dimethyl sulfoxide (DMSO) has been shown to improve the yield of the reaction [85], though it may not be very well tolerated by cells during *in vitro* screening, requiring purification to remove the solvent. We demonstrated a combinatorial approach to develop multiple derivatives of primary, secondary and tertiary amines on a hyaluronic acid backbone using an amidation reaction and have subsequently used a blend of these derivatives for the successful delivery of siRNA [86].

4.3.3 Click Synthesis: Click synthesis is not a single reaction but refers to a group of reactions. It was demonstrated for the first time by Dr. Sharpless in 1999 and has been explained by him as a group of reactions that are modular, wide in scope, high product yielding with easy

purification by crystallization or distillation, without offensive by-products and are stereospecific [87]. As elucidated from this definition, click chemistry fulfills all the necessary criteria that would be ideal for a synthetic scheme for combinatorial design of materials. Most importantly, click synthesis can be achieved in relatively mild reaction conditions in an aqueous environment. Therefore, it is not surprising that this method of synthesis has been explored for a wide variety of applications in pharmaceutical science[88] including combinatorial design of materials. However, click chemistry does have certain disadvantages as well. Involvement of copper as a catalyst has been one of the major criticism because presence of residual copper in the final product may not be well tolerated *in vivo* and can cause renal, hepatic or neurological toxicity [89]. Therefore, considerable effort has been made to develop copper-free methods for synthesizing compounds by click chemistry [90]. Abeylath *et al.* applied click synthesis to modify the backbone of dextran with varying lengths of lipid chains (C_2-C_{12}), thiol groups and PEG to develop a library of compounds that could successfully self-assemble to encapsulate drugs with logP values ranging from -0.5 to 3, confirming that optimum design and careful choice of material can aid in loading small drug molecules with varying solubility [91].

4.3.4 Dehydration Reaction: A dehydration reaction is typically referred to as a chemical reaction where the bond formation between individual reactants involves removal of water molecule. Dr. Kohn's lab has adopted a combinatorial strategy to develop polymeric derivatives using diphenolic monomers of amino acid L-tyrosine [92]. Esterification between an alcohol and an acid is one of the most commonly used dehydration reaction and has been efficiently used for combinatorial design of polymer libraries. Brocchini et al. developed combinatorial library of A-B type 112 polyarylates copolymers using 14 distinct tyrosine-derived diphenols and 8 aliphatic diacids [93]. Their design approach used a permutation and combination approach of monomers by varying the pendant group of diphenol block and the alkyl chain length of diacid block, thereby modulating the physical properties of the resulting copolymers and subsequently the structure property relationship [94]. Additionally, use of these natural metabolite monomers generates a library of highly biocompatible and biodegradable polymers, which were demonstrated for potential application in medical implants [95]. Similarly, phosgene mediated reaction between diols was demonstrated for formation of ether carbonates as a synthesis strategy to combinatorially generate copolymers with varying degree of molar percent fractions of diphenol monomers as well as their PEG derivatives [96, 97].

4.3.5 Enzymatic polymerization: Enzyme mediated synthesis of polymer is particularly attractive since the reaction is highly specific, can be performed in a much milder conditions in the absence of organic solvents or toxic chemicals and thus yield a more benign approach for development of biomaterials. One of the early reports on biocatalyzed synthesis of combinatorially designed polymer used lipase enzyme to design derivatives of lactones, divinyl esters and glycols [98]. The 12-, 13- and 16- carbon chain derivatives were used in this study where both polycondensation and ring-opening approach for polymer synthesis could proceed in one pot via a common acyl-enzyme intermediate. Following the cue from this study, Park et al. screened 3 proteases and 4 lipases for their enzymatic activity in different solvents to identify the ideal enzyme for enantio- and regioselective polymer synthesis. They demonstrated that Novozyme-435 (lipase) outperformed others with 20% transesterification of sugar in acetone while Opticlean M375 (protease) showed 55% transesterification capability in pyridine [99]. They further synthesized an array of monomers using four straight chain diesters as acyl donors (C_3-C_{10}) conjugated to aliphatic and aromatic diols, carbohydrates, nucleotides and natural steroid using lipase-mediated biocatalytic reaction [100]. The biocatalytic approach offers excellent stereo, region and chemo selectivity and therefore has been enthusiastically pursued and developed in the last decade [101-103].

5. Illustrative Examples in siRNA and Drug Delivery

5.1 Dextran-Based Nanoparticles

Dextran is a highly water-soluble branched polysaccharide made of glucose repeat units and has been extensively studied for drug delivery applications as a drug conjugate or nanoparticulate delivery system due to its high biocompatibility, biodegradability and non-toxicity [104]. Presence of multiple hydroxyl groups on the polymer backbone allows for suitable modification making them an ideal polymeric system for combinatorial design. Abeylath *et al.* applied click synthesis chemistry on the polymer backbone where the parent polymer was first converted to O-pentynyl dextran, which served as a precursor for subsequent synthesis steps to yield lipid, thiol or PEG derivatives of dextran (**Figure 4**). The O-pentynyl dextran was used as a starting material to synthesize a library of lipid derivatives with varying chain length ranging from C_2 to C_{14}. The lipid derivatives of dextran could readily self-assemble in an aqueous environment to form nanoparticles and encapsulate a drug in the process (**Figure 5**). Simultaneously, thiol

derived dextran derivatives could be used to cross-link nanoparticles by disulfide linkage to provide stability to the nanoparticles while PEG-dextran could provide surface stability and stealth characteristics to the nanoparticles in *in vivo* applications. A systematic study of drug encapsulation using model drugs with logP value in the range of -0.5 to 3 showed that hydrophilic drugs tend to show better encapsulation in nanoparticles formed using small lipid chain lengths while hydrophobic drugs encapsulated better in dextran nanoparticles modified with longer chains of lipids. It was further ascertained that the size of the nanoparticle showed strong dependence on the molecular weight of the dextran polymer where MW 10 kDa dextran formed smaller sized particles than the MW 40 kDa dextran for a similar lipid chain modification [91].

An ideal combinatorially designed library of material should show versatility in payload encapsulation upon careful choice of building blocks during nano-vector designing. Kobayashi *et al.* demonstrated that the various derivatives of dextran can be utilized not only for the delivery of small molecules but also for the delivery of small interfering RNA (siRNA) by careful choice and "mix and match" of derivatives in appropriate proportion [105]. Doxorubicin (DOX) as a model small molecule drug could be loaded in a blend of C_6 modified dextran, dextran-thiol and dextran-PEG to form self-assembled nanoparticles of 171 ± 2 nm size and a surface charge of -1.21 mV. Concomitantly, siRNA against multidrug resistance protein 1 (*MDR1*) could be encapsulated in octylamine-modified dextran, dextran-PEG and dextran thiol. The resultant self-assembled nanoparticles were found to be 101 ± 3 nm sized with a surface charge of -0.22 mV. *In vitro* cytotoxic evaluation of DOX loaded dextran nanoparticles in drug sensitive osteosarcoma and ovarian cancer cells (KHOS and SKOV3 respectively) showed significantly higher (5-10 fold) dose dependent anti-proliferative activity compared to free drug in solution as well as to DOX loaded in a liposomal formulation. Drug resistant osteosarcoma and ovarian cancer cells ($KHOS_{R2}$ and $SKOV3_{TR}$ respectively) showed higher resistance to free drug treatment as well as to treatment with drug loaded in a liposomal formulation. The anti-proliferative activity of DOX loaded in dextran nanoparticles was nearly two-fold higher than DOX loaded in the liposomal formulation, suggesting that dextran loaded nanoparticles show better drug delivery characteristics. Most importantly, blank dextran nanoparticles did not show any adverse effect on any of the four tested cell lines confirming that the individual building blocks and the nanoparticle system as a whole does not have substantial cytotoxic impact.

As a second arm of this study, we studied the nucleic acid transfection efficiency of self-assembled dextran nanoparticles. AF488-tagged *MDR1* siRNA loaded in dextran nanoparticles was transfected in drug resistant $KHOS_{R2}$ osteosarcoma cells where fluorescence microscopy demonstrated an enhanced uptake of the dextran-encapsulated siRNA within 2 h of incubation compared to free siRNA. The fluorescence intensity from nanoparticles was qualitatively higher than that from siRNA transfected with lipofectamine™ RNAiMax transfecting agent. A majority of delivery vectors successfully ferry nucleic acid payloads into the cells but the cargo fails to show any activity. An activity assessment therefore is essential to correlate increased siRNA uptake with its function and confirm that the nanoparticles can successfully release the siRNA efficiently after entering cells. P-glycoprotein (the translational product of the *MDR 1* gene) levels were evaluated in cells transfected with *MDR1* siRNA, which clearly indicated a significant knockdown in the protein level, thereby confirming that dextran nanoparticles were able to enter the cells and deliver the siRNA [105].

5.2 Hyaluronic Acid-Based Nanoparticles

Hyaluronic acid (HA or Hyaluronan) is another natural anionic polysaccharide containing repeat units of glucuronic acid and N-acetylglucosamine that has been used for the combinatorial design of a library of structurally unique polymers mainly due to its highly biodegradable, non-toxic, non-immunogenic and non-inflammatory properties. More importantly, HA has a natural tendency to recognize and bind to cluster of differentiation 44 (CD-44) receptors that are highly overexpressed on the surface of the majority of cancer cells [106]. The HA backbone contains a high density of carboxylic and hydroxyl groups that are amenable for chemical modification and have been extensively used as delivery vectors in the form of drug conjugates or nanoparticle delivery systems [107]. Even though there is a plethora of literature on their application in drug delivery and tissue engineering, the majority of the reported work is sporadic and a concerted effort on exploiting its potential has been lacking. We reported a combinatorial approach using straightforward yet versatile EDC/NHS coupling chemistry for synthesizing a library of amine derivatives of HA (MW 20 kDa) (**Figure 6**) [86]. Applying a systematic approach, the activated backbone of HA was functionalized with primary mono-functional fatty amines of the general formula $CH_3(CH_2)_nNH_2$ (n=3, 4, 5, 7, 9, 11, 13 and 17), bi-functional fatty amines of general formula $NH_2(CH_2)_nNH_2$ (n=4, 5, 6….) and polyamines such as polyethyleneimine (PEI, MW 10

kDa) and poly(L-lysine) (PLL, MW 10-14 kDa). Simultaneously, PEG (MW 2000 and 5000) residues were conjugated to impart surface stabilization and stealth character upon incorporation in to the nanoparticles. The amine derivative of HA was blended with PEG-HA and the material was tested for self-assembly to form nanoparticles, siRNA encapsulation efficiency, siRNA activity, nanoparticle size and charge. The initial screening confirmed that HA-PEI derivatives could successfully self-assemble and encapsulates siRNA in water or phosphate-buffered environment (N/P ratio 54:1) to yield sub-100 nm sized particles with a net negative charge. *In vitro* assessment further confirmed that these nanoparticles could successfully deliver the payload into human lung cancer cells by targeting the CD44 receptor that is over expressed on the surface of these cells. Among the various tested formulations, HA-PEI/HA-PEG nanoparticles outperformed and showed nearly 55% *in vitro* gene silencing, confirming that the nanoparticles could efficiently release the payload within the intracellular compartment to show desired activity [86].

We further studied the effect of combination treatment of HA-PEI/HA-PEG loaded siRNA against *survivin*, *bcl-2*, *mdr1* and *mrp1* genes with the small molecule anticancer drug cisplatin in human non-small cell lung cancer cells A549 and its cisplatin-resistant counterpart $A549^{DDP}$. The obtained *in vitro* cytotoxicity assessment confirmed that a combination of cisplatin along with siRNA against *survivin* and *bcl-2* resulted in nearly 80% cell death in cisplatin resistant $A549^{DDP}$ cells. This combination was further tested *in vivo* in $A549^{DDP}$ xenograft tumor bearing mice. For *in vivo* studies, cisplatin was encapsulated in octadecylamine modified HA nanoparticles. The *in vivo* efficacy assessment indicated that the combination treatment resulted in nearly 62% suppression in tumor growth compared to other treatment and control groups. Body weight of the mice, liver enzyme levels and immunohistochemistry did not show any abnormalities within the various treatment groups during the course of the therapy confirming that the delivery system did not show any apparent toxicity [108]. Qualitative biodistribution studies in A549, $A549^{DDP}$, H69 and H69AR xenograft tumor bearing mice using indocyanine green (ICG) loaded HA-PEI/HA-PEG nanoparticles showed that both A549 and $A549^{DDP}$ tumor bearing mice showed high accumulation of particles within 10 h of injection (**Figure 7**). The CD44 expression profile of both of these cells revealed a very high density of the receptor on the surface, which explains the receptor-mediated high accumulation of HA-PEI/HA-PEG nanoparticles. H69 and H69AR small cell lung cancer tumor bearing mice, on the other hand, showed poor tumor accumulation, which could be correlated to their low expression of the CD44 receptor (60 and 90% respectively). The gene

knockdown studies in these animal models showed a similar trend indicating that the penetration of the nanoparticles in low CD44 expressing cells is limited and that the HA-based delivery vector facilitated enhanced tumor penetration and subsequent activity of the payload owing to its ability to bind to the receptor[109]. Quantitative biodistribution studies in a non-small cell lung cancer $A549^{DDP}$ tumor xenograft model showed increased concentration of siRNA in the tumor when delivered by the nanoparticles, which is inline with the qualitative biodistribution and gene knockdown results. These results clearly indicate that an inherently targeted, modular platform can have a significant impact on improving the performance of the existing drugs as well as drug delivery systems.

5.3 Poly (β-amino ester)-Based Nanoparticles

Combinatorial design of a library of polymers on PBAE backbone was a technology that has been developed and extensively studied in Dr. Langer's lab. In early 2000, the first demonstration of parallel synthesis of polymer library was developed by chemical conjugation of primary and secondary amines to diacrylates by amine addition reaction [55]. Using 7 different diacrylates and 20 amines, a library of 140 unique polymers was generated from which 70 polymers showed acceptable aqueous solubility and were further screened for plasmid DNA encapsulation and transfection capability. The same group further developed a semi-automated high-throughput method to screen a library of 2350 structurally distinct polymers for gene delivery applications [56]. The synthesis scheme allows grafting of primary and secondary amines onto the poly(β-amino ester) (PBAE) backbone to yield highly biocompatible and biodegradable cationic polymers that could efficiently complex to negatively charged nucleic acid molecules for delivery applications. This methodology also permits the synthesis, storage and *in vitro* screening of the derivatives in the same reaction well without a need for the removal of solvents or purification of the product, thus being amenable to high-throughput automated screening. In fact, authors demonstrated the capability of synthesizing all 2350 polymer derivatives in a single day and could perform gene transfection studies at an astounding rate of 1000 per day using semi-automated methods [56]. Initial studies on nucleic acid transfection confirmed that 33 and 46 of the 2350 tested polymer showed transfection efficiency similar or better than PEI under non-optimized and optimized conditions respectively. Synthesis of 486 second-generation derivatives with careful consideration for structure-property relationship of the polymers for nucleic acid delivery, 20 of

the 70 unique primary structures used demonstrated transfection efficiency better than Lipofectamine 2000, commercial gold standard transfection agent [84]. Anderson et al. more recently demonstrated that the PBAE derivatives could be further photocrosslinked to improve the mass-profile, control the degradation behavior and impart better mechanical property to the nanoparticle system [110]. A focused detailed account on materials design considerations and developed methodologies for PBAE-based combinatorial library is highly recommended to the reader [111].

Process development, characterization and *in vitro* validation of the PBAE-based polymer library has been suitably followed with pre-clinical evaluation studies to confirm the capability of the delivery system to perform *in vivo*. Greenland et al screened potential candidates from the PBAE library to identify polymers that show enhanced *in vivo* transfection efficiency of plasmid DNA adjuvants for vaccine application [112]. Among the tested polymer members, poly [(1,5-di(acryloxyethoxy)hexane)-co-(4-aminobutanol) show the best activity with a seven fold increase in the gene expression and 70% enhancement in subsequent immune response in Balb/c mice. Based on the results obtained from screening the polymer library for gene delivery, authors could also conclude that in general, polymers with a moderate hydrophobic backbone were more effective in transfection efficiency *in vivo*. Possibility of such rigorous experimental analysis of structure-property relationship is the biggest advantage of combinatorial approach of material design.

6. Conclusions and Future Outlook

Combinatorial design of materials for developing novel drug delivery systems is an emerging and powerful approach, which is still in its infancy and its true potential is yet to be unveiled. The conventional approach of polymeric drug delivery based on “one polymer at a time” has been proven to be uni-dimensional, time consuming, labor intensive and economically inefficient. Conversely, a combinatorial approach offers tremendous promise as a versatile and customizable method where the properties of the delivery vector can be tailored at will to make them amenable to the delivery of small molecules, nucleic acid, peptides or protein based therapeutic agents. To this end, the past decade has seen some encouraging advances in developing libraries of combinatorially designed compounds that can be selectively picked and mixed and matched to provide desired properties to a delivery vector. Choice of simple yet elegant synthesis

methods has allowed minimal post-synthesis processing of the products, thus making the approach amenable to high-throughput screening for rapid output. Polymers have been most popularly explored for such applications since they enjoy a rich history as materials for drug delivery. They are generally biocompatible and biodegradable and the multiple repeats of monomers provide abundant functional groups for chemical modification. *In silico* simulations based on existing knowledge of material and drug properties particularly assist in predicting the material-drug, material-material and drug-drug interactions, which eventually forecasts the drug encapsulation and release in a particular environment. Advances in chemical synthesis allowing precise and controlled grafting on the polymer backbone, sophisticated methods for purification and characterization of these derivatives, fairly understood properties of the payload as well as improved understanding of cancer biology and physiology further aid in designing materials. Therefore, a collaborative effort of bioinformatics, polymer chemistry, pharmacology, cancer biology and instrumentation has immensely benefitted the field of combinatorial design of materials with some successful *in vitro* and *in vivo* research.

Dr. Langer's group has tremendously contributed to the development of this approach and many other research labs are now actively pursuing research on the combinatorial design of materials for drug delivery applications and beyond. Hook *et al.* recently demonstrated that careful selection of structurally related materials from a library of products could be used as a protective coating on the surface of medical implants[113]. Riding on the glory of such success stories, combinatorial approach for material synthesis is enjoying considerable attention but a careful assessment reveals that the existing knowledge pool is limited to a few selected examples. Current strategies rely on a few synthesis strategies that offer limited application and therefore, there is a need to develop new methods that do not use harsh procedures, can be easily scaled up and provide consistent products with minimum batch to batch variation. Besides, the majority of the efforts have been driven by the need for effective delivery vectors for extremely labile therapeutic molecules such as genes, siRNA and miRNA and therefore almost all reports focus on grafting positively charged amine groups on polymeric backbones for subsequent electrostatic complexation with negatively charged nucleic acids. However, the diverse physical properties of small molecule drugs have largely been ignored where a modular platform developed on a common backbone will greatly benefit formulation design and development. The benefits of combination therapy using RNA interference (RNAi) and anticancer drug in overcoming MDR has

been well acknowledged and the flexibility of using structurally similar delivery systems for both types of therapeutic moieties certainly makes an exciting prospect; highlighting the demand for successful combinatorial approaches.

Material safety is another important criteria that has to be carefully studied. Combinatorial synthesis schemes generally start with a careful choice of a parent polymer backbone, which has a well-characterized profile of biocompatibility, biodegradability and clearance. However, the tolerance of chemical ligands and various solvents used in the chemical synthesis or that of the modified polymer itself is poorly understood. This aspect gains more traction due the fact that the primary aim of combinatorial chemistry is to develop a library of materials with diverse structure-property relationships. While the majority of endeavors focus on adopting reproducible synthesis approaches, high-throughput screening for suitable "hits", and subsequent applications in designing novel delivery vectors, an emphasis on the safety of these materials is often ignored. United States Food and Drug Administration (FDA) has exercised strict guidelines that have to be met before any delivery system can transition from the laboratory to clinical level and safety is a key parameter that must be profiled for approval. Therefore, much work is needed before this promising area of research can meet its potential but the initial success certainly indicates an encouraging future for this approach.

7. References

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Figure Captions

Figure 1: *Schematic illustration of DOX-loaded zwitterionic polymer-based NPs and the changing of surface charge property in response to extracellular pH. (1) Amphiphilic zwitterionic block copolymer PCL-b-P(AEP-g-TMA/DMA) self-assembles into NPs in aqueous solution with DOX encapsulation. During circulation in blood, the NPs show prolonged circulation time and can leak into tumor sites through the EPR effect. (2) Responding to the extracellular pH, the zwitterionic polymer diminishes its anionic part, forming PCL-b-P(AEP-g-TMA/Cya), and the formed NPs are activated to be positively charged and become recognizable by tumor cells. Adapted from Yuan et al., 2012,[40] Copyright 2012, with permission from WILEY-VCH.*

Figure 2: *Schematic Depiction of Nanoparticles Coated with Multilayer Shells as New Drug Delivery System. The multifunctionality arises from the stepwise construction of the shell that is assembled by the layer-by-layer (LBL) method. The internal layers are split in two compartments (yellow (1) and red (2)) in order to indicate that different functionalities can be integrated in a modular way in different layers. The yellow compartment (1) serves primarily to compatibilize between the core and the external layers. The yellow and the red compartment can incorporate drugs, radionuclide for radiotherapy, proteins/nucleotides for bioactivity, or contrast agents for detection. The external layers carry functionalities such as enzymatically cleavable drugs or ligands for receptor mediated targeting, both of which must be accessible on the outside. Reprinted from Schneider et al., 2009[52] with permission from ACS publication.*

Figure 3: *Instantaneous drug release profile per unit area of a film as predicted by Higuchi equation.*

Figure 4: *Schematic representation of the synthesis approach for alkyl-modified (6), thiol-modified (7) and PEG-modified (8) derivatives of dextran using Click chemistry. Reprinted from Abeylath et al., 2011[91] with permission from Elsevier.*

Figure 5: *Schematic illustration depicting formation of drug (1) encapsulated self-assembling nanoparticles using dextran building blocks modified with lipid chain (2), thiol (3) and PEG (4). Reprinted from Abeylath et al., 2011[91] with permission from Elsevier.*

Figure 6: *Scheme representing the methodology adopted for synthesis of amine derivatives of Hyaluronic acid (1). (2) HA conjugated to monofunctional fatty amine. (3) PEG derivative of HA. (4) HA grafted with bifunctional fatty acid. (5) Thiol derivative of HA. (6) Polyamine derivative of HA. All the synthesis chemistry follow simple yet versatile EDC/NHS coupling. Reprinted from Ganesh et al., 2013[86] with permission from Elsevier.*

Figure 7: *Qualitative biodistribution analysis of indocyanine green encapsulated HA-PEI/HA-PEG nanoparticles in human non-small cell lung cancer A549 and $A549^{DDP}$ (A) and small cell lung cancer H69 and H69AR cells (B) tumor bearing mice. Free indocyanine green dye was injected as control to see clearance of dye from the circulation in mice (C). The images have been acquired using IVIS live imaging station. Reprinted from Ganesh et al., 2013[109] with permission from Elsevier.*

Figure 1

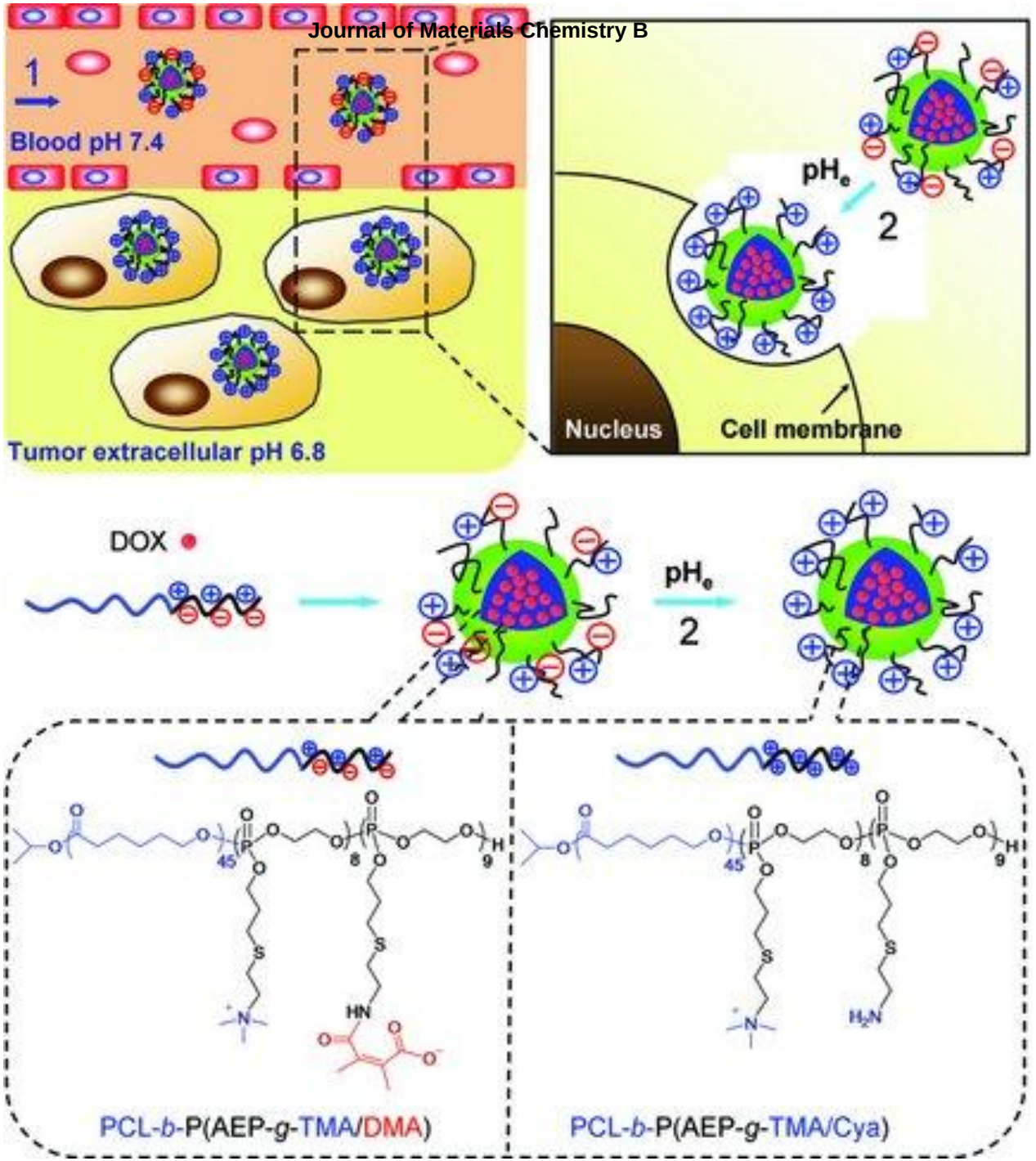

Figure 2

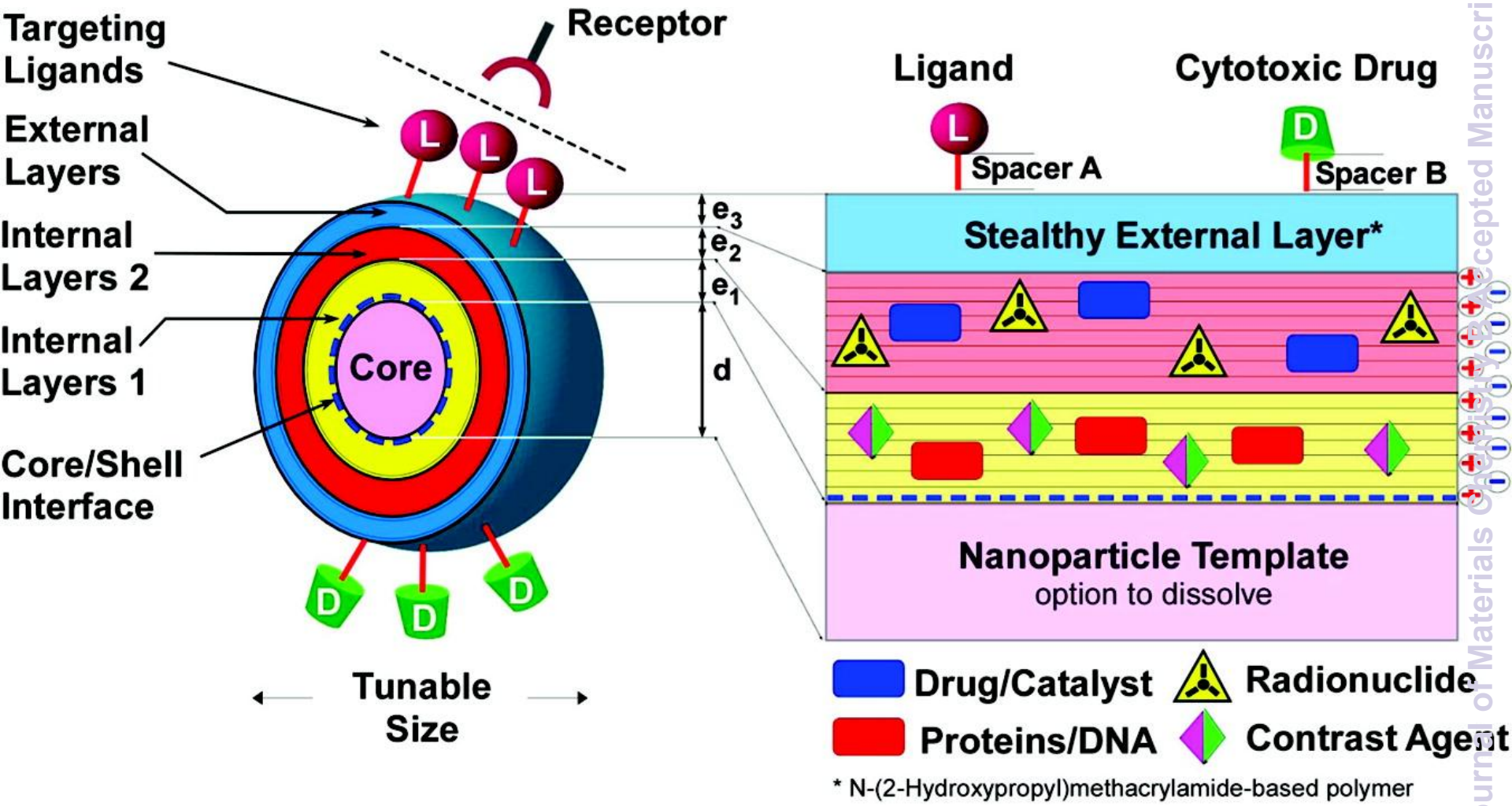

Figure 3

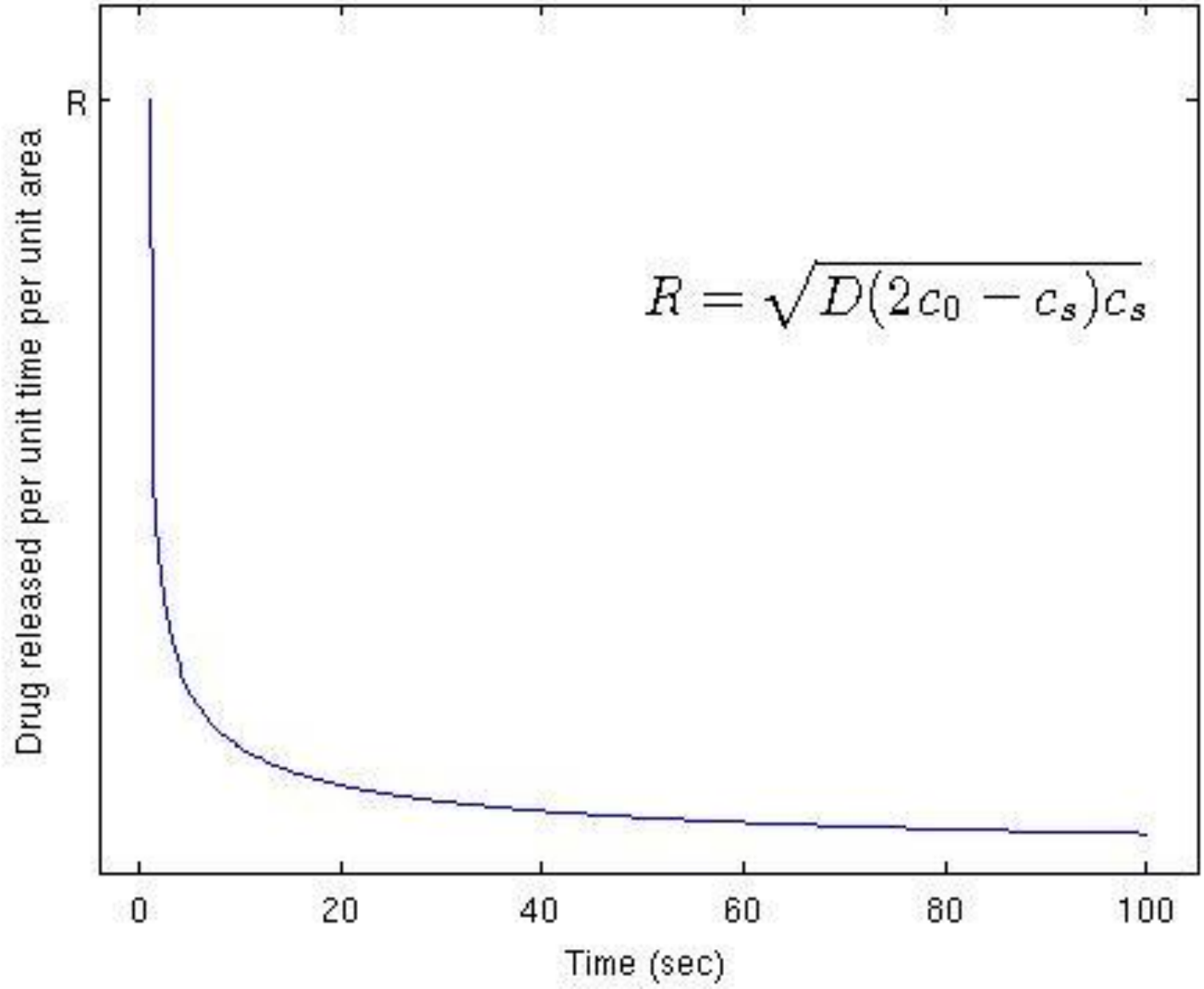

Figure 4

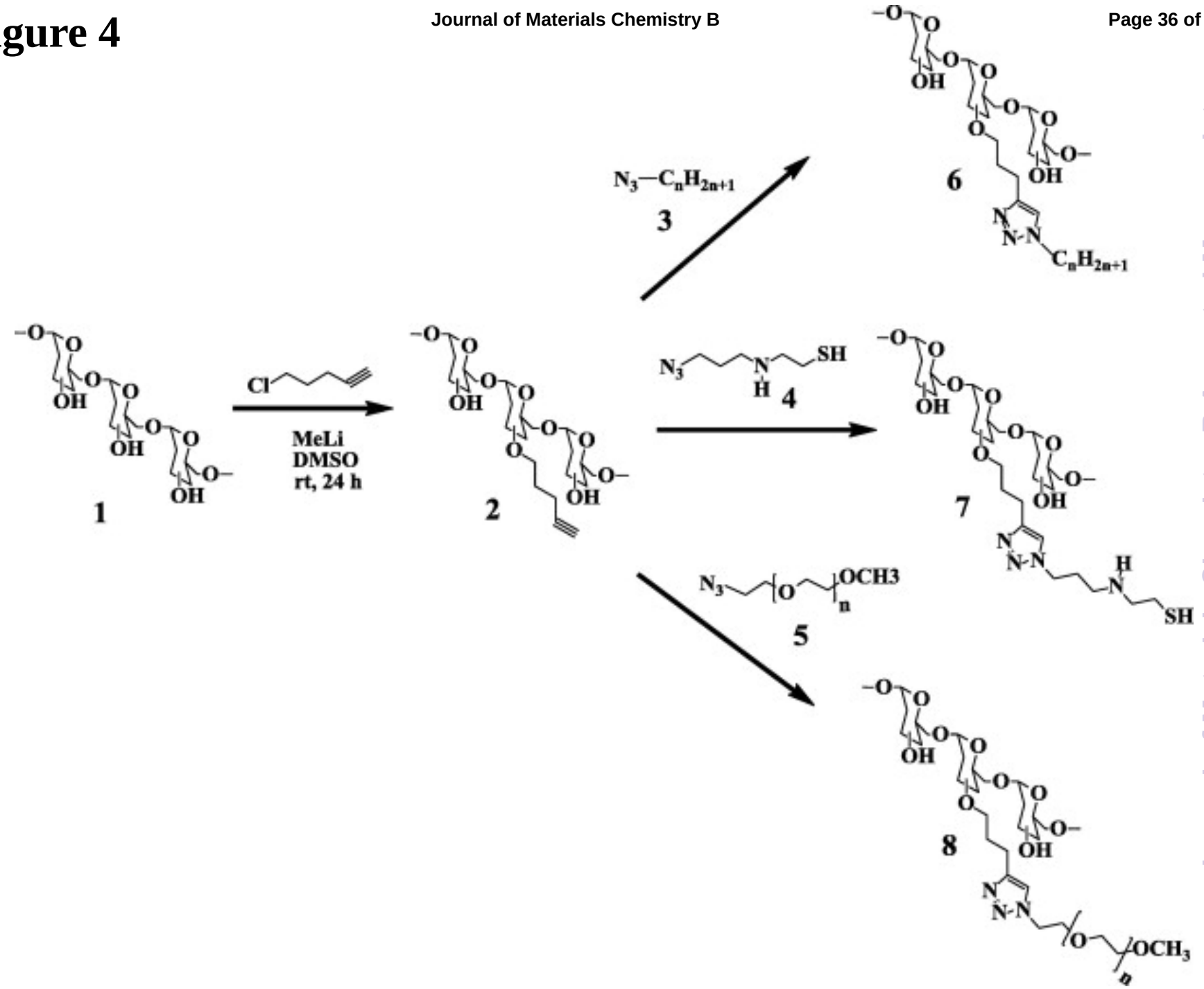

Figure 5

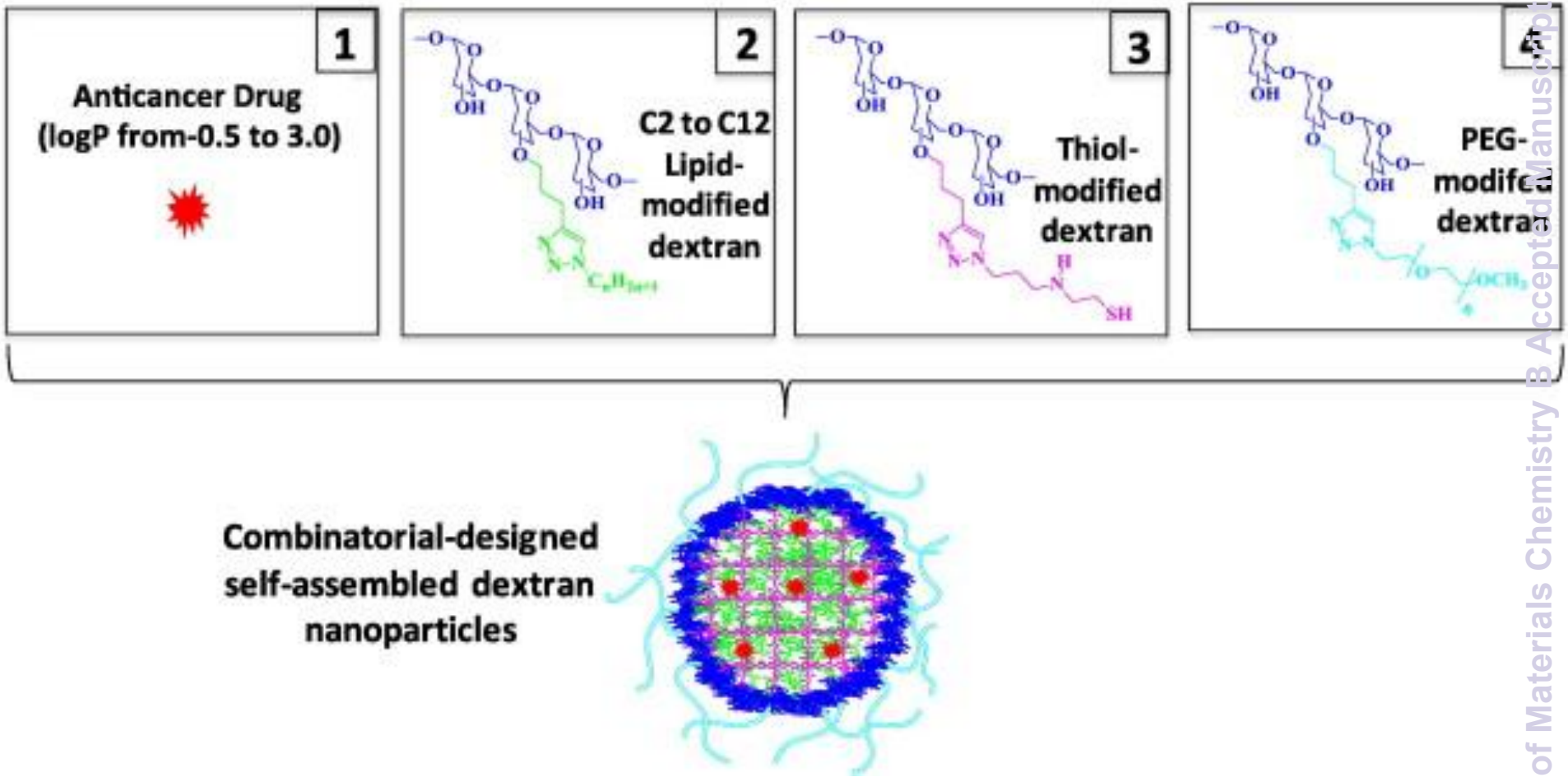

Figure 6

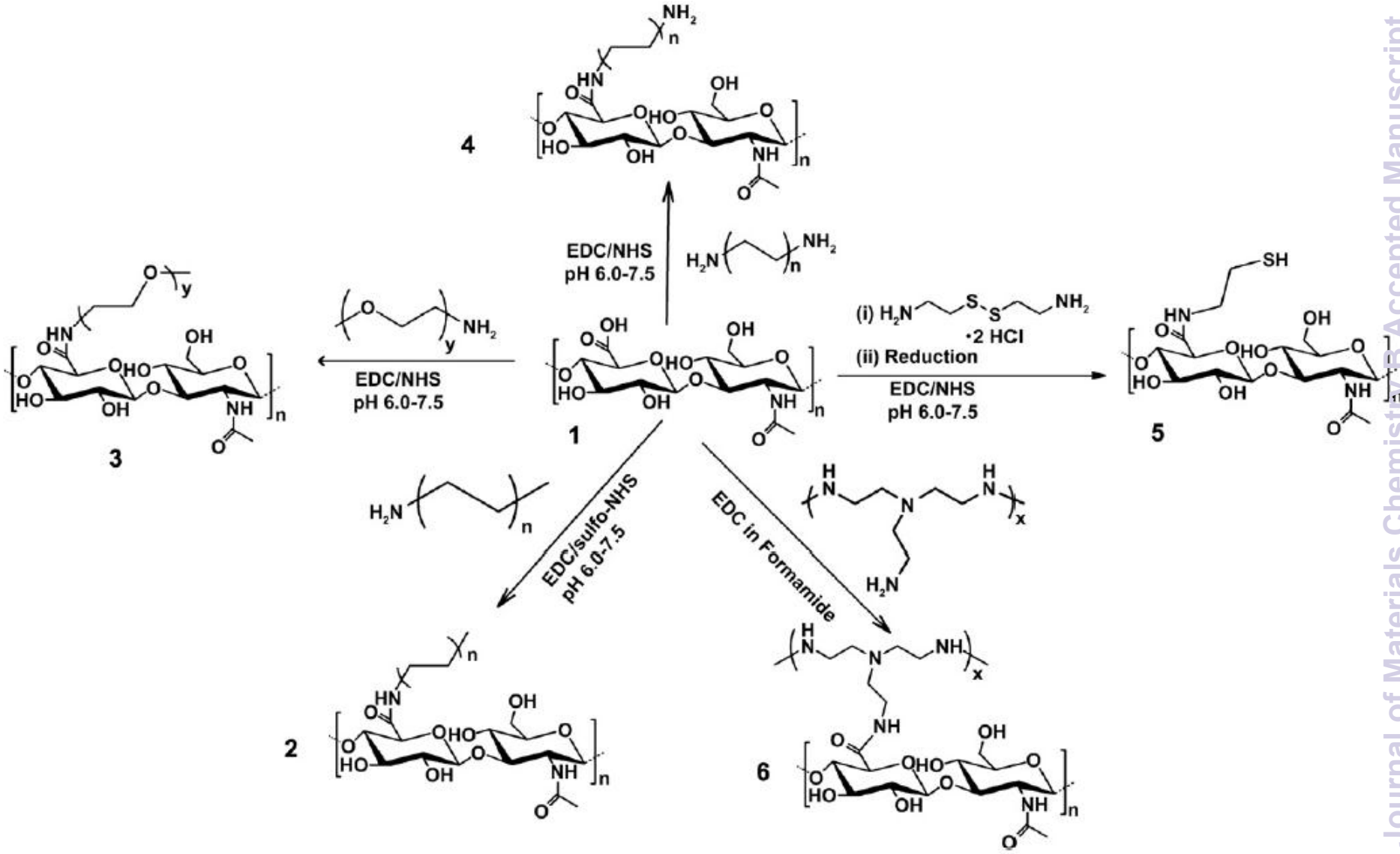

Figure 7