DESIGN AND SYNTHESIS OF MULTIFUNCTIONAL POLY(ETHYLENE GLYCOL)S USING ENZYMATIC CATALYSIS FOR MULTIVALENT CANCER DRUG DELIVERY

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DESIGN AND SYNTHESIS OF MULTIFUNCTIONAL POLY(ETHYLENE GLYCOL)S USING ENZYMATIC CATALYSIS FOR MULTIVALENT CANCER DRUG DELIVERY

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ABSTRACT

The objective of this research was to design and synthesize multifunctional poly(ethylene glycol)s (PEG)s using enzyme-catalyzed reactions for multivalent targeted drug delivery. This approach was intended to address the design and synthesis flaws of folate-targeted dendrimers developed by University of Michigan researchers. The cancer targeted nanodevice based on generation 5 poly(amidoamine) G5 PAMAM dendrimers conjugated to an average ~ 5 folic acid (FA) targeting ligands demonstrated an enhanced binding to KB tumor cells and targeted KB cells preferentially in \textit{in vitro} and \textit{in vivo} studies. However, the device showed two major flaws: structural defects in the PAMAM dendrimer and heterogeneous ligand distribution.

Based on computer simulation for optimum folate binding, a four-arm PEG star topology with $M_n = 1000$ g/mol was proposed. First, a four-functional core based on tetraethylene glycol (TEG) was designed and synthesized using transesterification and Michael addition reactions in the presence of \textit{Candida antarctica} lipase B (CALB) as a biocatalyst. The four-functional core $(\text{HO})_2$–TEG–(OH)$_2$ core was successfully prepared by the CALB-catalyzed transesterification of vinyl acrylate (VA) with TEG and then Michael addition of diethanolamine to the resulting TEG diacrylate with/without the use of solvent.
The functional PEG arms with fluorescein isothiocyanate (FITC) and folic acid (FA) were prepared using both traditional organic chemistry and enzyme-catalyzed reactions. FITC was reacted with the amine group of H$_2$N-PEG-OH in the presence of triethylamine via nucleophilic addition onto the isothiocyanate group. Then, divinyl adipate (DVA) was transesterified with the FITC-PEG-OH product in the presence of CALB to produce the FITC-PEG vinyl ester that will be attached to the four-functional core via CALC-catalyzed transesterification. For the synthesis of FA-PEG vinyl ester arm, DVA was first reacted with PEG-monobenzyl ether (BzPEG-OH) in bulk in the presence of CALB. The BzPEG vinyl ester was then transesterified with 12-bromo-1-dodecanol in the presence of CALB. Finally, BzPEG-Br was attached to FA exclusively in the gamma position using a new method.

The thesis also discusses fundamental studies that were carried out in order to get better understanding of enzyme catalyzed transesterification and Michael addition reactions. First, in an effort to investigate the effects of reagent and enzyme concentrations in transesterification, vinyl methacrylate (VMA) was reacted with 2-(hydroxyethyl) acrylate (2HEA) in the presence of CALB. When the reaction was performed in tetrahydrofuran (THF) with a 2HEA concentration of 0.10 mol/L, only 19% conversion was observed within 4 hours, whereas complete conversion was achieved under solventless conditions. The effect of enzyme concentration in reactions with and without solvent was also studied. Quantitative conversion was achieved within 4 hours with [CALB] = 2.0 × 10^{-4} mol/L in bulk, with no further rate increase with increased enzyme concentration. CALB showed high chemoselectivity in the reaction of DVA with 11-mercapto-1-undecanol in THF. DVA reacted only with the hydroxyl group of 11-
mercapto-1-undecanol while the thiol group remained intact. The regioselectivity was also studied by the CALB-catalyzed Michael addition of diethylamine to α-acrylate-ω-methacrylate and α-acrylate-ω-crotonate ethylene glycol which were prepared by the transesterification of VA, VMA and vinyl crotonate with 2HEA, respectively, under solventless conditions. Diethylamine exclusively reacted with the acrylate group of both compounds. The Michael addition of diethanolamine to TEG dimethacrylate only gave 12.1% conversion within 24 hours and no further progress was observed after 24 hours. In contrast, TEG diacrylate reacted completely with diethanolamine within 2 hours.

The effect of DVA concentration on the CALB-catalyzed transesterification with TEG was studied under solventless conditions. When 1.5 molar equivalent of DVA per OH in TEG was used, 42% divinyl-functionalized product was observed together with 56.5% oligomerized (di-, tri-, tetra- and pentamer) products. At 10 eq. of DVA, only 18.4% oligomerized products were obtained. The effect of diol molecular weight was also investigated. At 10.0 eq. DVA per OH only 2% dimer was observed with PEG $M_n=1000$ g/mol, and a single divinyl functionalized product was obtained with $M_n=2000$ g/mol. The effects of polymer molecular weight and DVA concentration were also studied in the reaction of DVA with PEG monomethyl ether (MPEG-OH, $M_n=1100$ g/mol and 2000 g/mol). The extent of coupling decreased from 35% to 0.4% when the DVA concentration was increased from 1.5 to 10 per –OH in the MPEG-OH. No coupling was observed with MPEG-OH $M_n=2000$ g/mol at 5 eq. DVA per –OH.

Following these fundamental studies, TEGs and PEGs were enzymatically functionalized. TEGs were transesterified with VMA and vinyl crotonate in the presence of CALB under solventless conditions within 4 hours of reaction time. Benzyl protected
TEG-OHs were also successfully functionalized with VMA and vinyl crotonate in the presence of CALB under solventless conditions within 2 hours. An eight-functional molecule was also synthesized from (HO)₂⁻TEG⁻(OH)₂. First an α-vinyl-ω-acrylate linker was prepared by the transesterification of DVA with 2HEA. This linker was then transesterified with (HO)₂⁻TEG⁻(OH)₂, followed by Michael addition of DEA to the tetra-acrylated TEG.

PEGs were also successfully functionalized by the enzymatic transesterification using vinyl esters such as VMA, vinyl crotonate and VA under solventless conditions within 4 hours. PEG diacrylate was further functionalized by the Michael addition of DEA, resulting in a tetra-hydroxy functional PEG [(HO)₂⁻PEG⁻(OH)₂].

The structure of all products was confirmed by $^1$H NMR, $^{13}$C NMR and MALDI-ToF MS.
DEDICATION

This dissertation is dedicated to my family: my father, Young Sun Seo; my mother, Jung Yub Kim; my brother, Kwang-Heue; and my wife, Nan Young Kim as well as my angels, Chansuk and Chanyoo for all of their love and support.
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CHAPTER I
INTRODUCTION

Multivalent binding of cell membrane receptors using macromolecules offers a unique ability to target particular cells or pathogens with therapeutic agents.\textsuperscript{1-16} For cancer therapeutics, it is desired to deliver drugs directly to the cancer cell and kill it, while minimizing the collateral death of healthy cells. The controlled presentation of multiple targeting ligands by a single macromolecule is commonly referred to as multivalent targeting. The combined delivery of diagnostic and therapeutic agents is often referred to as “theranostics”,\textsuperscript{17} encompassing the development of novel approaches to deliver combined diagnostic and therapeutic agents to appropriate sites with high specificity and in adequate concentrations to realize the promise of combined diagnosis and treatment of diseases in a single molecule.

Researchers at the University of Michigan (UM) have previously demonstrated that generation 5 poly(amidoamine) (PAMAM) dendrimers with 128 $\text{\textendash}\text{NH}_2$ groups in the outer corona conjugated to an average of $\sim$5 folic acid (FA) targeting ligands and $\sim$5 methotrexate (MTX) drug molecules\textsuperscript{18,19} have an enhanced binding to KB tumor cells (human epidermoid carcinoma that overexpresses folate receptors)\textsuperscript{20}, and target KB cells preferentially in cell culture\textsuperscript{16,21} and in xenograft tumors in mice (Figure 1.1).\textsuperscript{22}
This G5 delivery device reduced tumor volume by a factor of two as compared to the equivalent dose (5.0 mg/kg) of free MTX or control saline injections. A similar tumor volume reduction was attained by employing 21.7 mg/kg of free MTX but this resulted in animal death within 30 days. The dramatic difference in the overall health of the animals during these treatments is illustrated in Figure 1.2. The mice with xenograft KB tumors receiving free MTX lost all of their hair and appeared sick. The mice receiving MTX conjugated to the targeted PAMAM retained all their hair and acted healthy.
The success of these targeting experiments both in vitro and in vivo prompted detailed studies to test the hypothesis if the targeted polymers actually functioned via a multivalent interaction. Quantitative measurements of the equilibrium constant $K_D$ with surface immobilized folate binding protein (FBP) using surface plasmon resonance (SPR) showed that the PAMAM-conjugated FA exhibited a ~2,500–~170,000 fold enhancement of binding avidities as compared to free FA. The origin of the exponential change in $K_D$ was found to arise from an exponential decrease in the dissociation rate ($k_{off}$) of the material as a function of the number of folic acid ligands attached, up to an average of ~5 FA. This multivalent FA system has also been employed for targeted delivery of iron oxide imaging agents and the same concepts have been extended to RGD and HER2 targeting ligands. The approach relies on presenting an optimized arrangement of targeting ligands to a set of receptors on the cell surface. However, UM researchers established that the multivalent PAMAM dendrimer contains two basic design flaws:
1. The polymer has a distribution of structures, with an average of $128 \pm 20$ –NH$_2$ groups in the outer corona;

2. The targeting ligands are conjugated to the dendrimer in a stochastic fashion, generating a broad distribution of the ligands conjugated per dendrimer.

Despite the great promise of the basic science achieved to date, substantial engineering challenges associated with materials synthesis have made translation to the clinic difficult to achieve. These challenges are succinctly summarized as 1) synthesis of the PAMAM conjugates with a sufficient degree of molecular structural control at the required scale and 2) identification of the design criteria that control multivalent binding.

The G5 PAMAM dendrimer theoretically contains 128 amine functional groups for conjugation. Even though the amine-terminated surface of PAMAM dendrimer allows for easy surface modification, the surface amine groups which are not involved in the conjugation with bioactive molecules have to be neutralized or appropriately modified to avoid toxicity of amine groups.$^{28-30}$ Upon conjugation of the ~5 FA targeting ligands, a broad distribution of species that contains dendrimers with 0 to ~20 FA attached is obtained.$^{31}$ The fraction of the population with the average number of 5 FA attached is quite small (<10%). For example, HPLC analysis of a dendrimer sample with ~2.7 FA measured by $^1$H NMR showed that 16% had no targeting ligand, and 22% contained only one targeting ligand. Thus ~40% of the sample would have no multivalent binding. This stochastic distribution of products applies to all polymer platforms containing functional groups and is not a problem unique to PAMAM dendrimers. However, this problem does
cause significant difficulties in obtaining a homogeneous targeted delivery agent. It also raises the question of what part of the 0-20 FA/particle distribution is the biologically active component. Furthermore, the chemistry developed to date is complex and has been difficult to scale to the kilogram range needed for human trials. The complexity of the conjugate mixtures also limited the conclusions that could be drawn regarding design optimization for multivalency. To address this basic flaw, we propose to synthesize PEG-based nanodevices for UM to test: “ideal” structures using enzymatic catalysis and attach FA, MTX and FITC to a multifunctional core.

![Figure 1.3. Ideal multifunctional structure.](image)

To avoid the cytotoxicity problem in an engineered platform, PEG was proposed as a building block. PEG polymers share many of the beneficial properties of PAMAM dendrimers, namely water solubility and resistance to recognition by the immune system.\(^ {32,33}\) PEG is one of the most widely used polymers in biomedical applications.\(^ {34,35}\) However, we realized that the basic design flaw related to the PAMAM dendrimers would also surface with dendrimers based on PEGs produced by “traditional”
conjugation methods. Any therapeutic platform conjugated with bioactive molecules such as drugs or targeting moieties by an excess of attachment sites can generate high degree of heterogeneity in the product. Thus we realized that we need to have simple and concise structure to well understand the variation in biological activity and to simplify the manufacture. In order to design and synthesize such a device, we proposed to apply new approaches to build our nanodevice based on well-defined building blocks.

My research advisor, Professor Judit E. Puskas and her previous student, Dr. Mustafa Y. Sen have developed the quantitative functionalization of various polymers, including a series of PEGs, using *Candida Antarctica* lipase B (CALB) as an enzyme catalyst. Enzymes are Nature’s catalysts that utilize specific reactions taking place in living systems. Enzymatic catalysis has been applied to polymer synthesis and functionalization with several advantages, including high efficiency, recyclability, the ability to operate under mild conditions, and environmental friendliness. Specifically, enzyme-catalyzed functionalization under neat condition, avoiding the use of organic solvents, is of great advancement in the design of a green process for biomedical application where the toxicity of solvents and catalyst residues need to be considered. Before our group had started working on the functionalization of preformed synthetic polymers, only a few examples were available in the literature with low conversions.

Based on attractive polymer functionalization methods using enzyme catalysis, we propose to synthesize multifunctional PEG macromolecules with precisely controlled numbers of arms containing targeting ligands and fluorescent dyes, and cancer drugs.
CHAPTER II
BACKGROUND

The objective of this research was the synthesis of multifunctional poly(ethylene glycol)s using enzyme catalysis for biocompatible multivalent targeted drug delivery. The background necessary to understand this research is detailed in the sections below.

2.1. Polymeric Nanomedicine: Drug Delivery Systems

Nanomedicine, integrating nanotechnology with medicine, is a relatively new subdiscipline. It deals with nanometer sized (100 nm or less) drug delivery systems designed for diagnostics and therapeutics with precise and efficacious treatments.\textsuperscript{48} The design of devices that can deliver sufficient drugs to targeted disease sites by overcoming the obstacles in physiological barriers and maintain low toxicity with efficient cure is one of the big challenges in drug delivery. Nanomedicine has the potential to provide beautiful solutions to the existing limitations of drug delivery systems. Specifically, one of the main approaches of nanomedicine is to create multi-functionality to drug carriers for improving the productivity of therapeutic drug treatment in a time and disease-specific manner.\textsuperscript{49,50}
Polymers play crucial roles in the design of nanocarriers for disease treatment; the term “polymeric nanomedicine” has been coined. Polymeric nanodevices with therapeutic agents can have a variety of different architectures, which are based on an original architecture of the macromolecules. Polymer-based drug carriers can be categorized by their primary functions (Figure 2.1).  

Figure 2.1. Schematic illustration of various polymeric nanomedicine drug delivery systems. Reprinted with permission from Polym. Rev. 2007, 47, 345-381. Copyright © 2007 Taylor & Francis.
Polymer drug conjugates: The conjugation between small molecule drugs and bio-engineered polymers has actively been pursued for improved pharmacological and pharmacokinetic properties of the therapeutic molecules as multifunctional nanodevices. In general, polymers provide increased aqueous solubility, biocompatibility, and prolonged plasma circulation half-life compared to free drugs. By applying targeting moieties, the polymer-drug conjugates can provide site-specific treatment with increased local drug concentration at the disease site.

Polymer-protein conjugates: A covalent link of hydrophilic polymers and protein therapeutics to form polymer-protein conjugates is the most widely adopted strategy to overcome pharmacokinetic and pharmacological drawbacks such as short circulating half-lives, immunogenicity, instability against proteolytic degradation, and low solubility. The manipulation of the amino acid sequences in protein therapeutics to reduce immunogenicity and improve stability has also been reported.52 There are three conditions for optimizing polymer-protein conjugations: (1) single linkage between polymer and protein to prevent the cross-linking of the protein, (2) stable and biocompatible conjugation chemistry to avoid immunogenic recognition and (3) reproducibility of protein modification through site-specific bioconjugation.53,54

Polymeric micelles: Amphiphilic block copolymers can form hydrophobic cored micelles in aqueous solutions above the critical micellar concentration. The inner core can serve as the “nanocontainer” of poorly water-soluble drugs.55 By applying bioactive agents on the outer shell, polymeric micelles can be used for targeting and/or imaging multifunctional devices for clinical use.56,57
Polymeric Vesicles: Polymeric vesicles can form liposome-like structures with a hydrophobic polymer membrane and hydrophilic inner cavity when the ratio of the hydrophobic domain to the hydrophilic domain is controlled within a certain range.\textsuperscript{58} Block copolymers self-assemble into vesicles by forming bilayers through the close packing of lipid-like, amorphous polymer hydrophobic segments, similarly to phospholipids. Polymeric vesicles are more stable than liposomes because of strong hydrophobic interactions between long hydrocarbon segments in the polymer chains.\textsuperscript{59,60}

Nanoparticles: Nanoparticles are submicron (< 1 µm) colloidal systems generally made of polymers. Based on the preparation procedure, nanoparticles can be categorized into nanospheres and nanocapsules. Unlike nanocapsules, which are vascular systems containing an aqueous or oily drug encapsulated in a core surrounded by a polymeric membrane, nanospheres are matrix systems in which the drug is dispersed in a polymeric matrix.

Dendrimers: Dendrimers are a class of well-defined macromolecules with nearly monodisperse, highly branched and globular-shaped nanostructures with large numbers of functional end groups and a size range of 0.1~20 nm in diameter. Dendrimers contain layered structures that extend outwards to multifunctional end groups, which can be modified or conjugated with bioactive molecules for drug delivery.

Although polymeric nanomedicine is a recent concept\textsuperscript{61-63}, polymer chemistry has been applied quite successfully as a methodological approach. Both natural and synthetic polymers\textsuperscript{50} have been used as drug carriers for decades. Most natural and synthetic polymers can be engineered to adapt a very specific function for a broad range of applications based on advanced nanotechnology; examples are shown in Table 2.1.
Table 2.1. Polymers used for drug delivery.

<table>
<thead>
<tr>
<th>Polymer types</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Natural</strong></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>Collagen, Albumin, Gelatin</td>
</tr>
<tr>
<td>Polysaccharides</td>
<td>Chitosans, Agarose, Alginate, Cyclodextrins, Polysaccharide Hydrogel</td>
</tr>
<tr>
<td><strong>Synthetic</strong></td>
<td></td>
</tr>
<tr>
<td>Biodegradable</td>
<td></td>
</tr>
<tr>
<td>Polyanhydrides</td>
<td>Poly(sebacic acid), poly(adipic acid), Poly(terephthalic acid)</td>
</tr>
<tr>
<td>Polyesters</td>
<td>Poly(lactic acid), Poly(glycolic acid), Poly(hydroxyl butyrate), poly(dioxanones)</td>
</tr>
<tr>
<td>Polyamides</td>
<td>Polyamino acids, poly(imino carbonates)</td>
</tr>
<tr>
<td>Non-biodegradable</td>
<td></td>
</tr>
<tr>
<td>Acrylic polymers</td>
<td>Poly(methyl methacrylate), Poly(hydroxyethyl methacrylate)</td>
</tr>
<tr>
<td>Silicones</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>Cellulose derivatives</td>
<td>Cellulose acetate, Hydroxypropyl methyl cellulose, Carboxymethyl cellulose, Ethyl cellulose</td>
</tr>
<tr>
<td>Others</td>
<td>Poly(ethylene glycol), Poly(vinyl pyrrolidone), Poly(ethyl vinyl acetate)</td>
</tr>
<tr>
<td>Dendrimers</td>
<td>Poly(amidoamine), Poly(propylene imine), Aromatic ester &amp;ether-type dendrimers</td>
</tr>
</tbody>
</table>

We will discuss dendrimers pertinent to this thesis in more detail.

2.2. Dendrimers

The dendritic structure is one of the topologies occurring in nature where a unique property needs to be enhanced. Examples may be found in both biological (tree and root branching, bronchioles and alveoli in lungs, neurons) and non-biological systems (e.g. snowflakes, drainage pattern, and lighting pattern). The exact reasons for the conformation of these dendritic topologies in nature are not entirely clear. However, one might speculate that the performance of a certain function can be exceeding the sum of
single applications if it is carried onto the surface of a dendritic pattern (synergistic effect).

In organic chemistry, dendrimers are a unique class of polymeric macromolecules that play a crucial role in the creative field of nanotechnology. Dendrimers are polymeric macromolecules with a regular and highly branched three dimensional architecture with low polydispersity and high functionality. A general dendrimer is composed of a core with several internal layers (called generation) and multi-functional surface groups (Figure 2.2).

Figure 2.2. Schematic diagram of a dendrimer structure.

The exact topology of a dendrimer depends on its chemical composition and the number of generations as well. The distinct features of dendrimers are amplified by increasing the generation number which affects three main “compartments”: the core, the outer shell and the surface.
The core: The core unit is surrounded by the dendritic wedges of increasing dendrimer generations. This creates cavities around the core into which small molecular drugs can be loaded.65

The outer-shell: The outer-shell has a well-defined microenvironment and is suitable for host-guest interactions and catalysis.

The surface: A high number of functionalities in the multivalent surface layer are available to conjugate bioactive agents, such as drugs, targeting moieties, imaging molecules, etc.

The desired molecular property or specific functionality can be obtained by designing these three compartments of the dendrimer.

2.2.1. The history of Dendrimer Design

Vögtle et al. (1978) described a series of synthetic “cascade” molecules as the first representatives of compounds exhibiting potentially perpetual branching.66 The cascade synthesis was performed by amidation of a difunctional nitrile, followed by Michael addition of acrylonitrile (Figure 2.3). The cycle can then be repeated perpetually. This basic concept led to the synthesis of various dendrimers using two major methods: the divergent and the convergent approach.

![Figure 2.3. Cascade synthesis by Vögtle and coworkers (1978).](image-url)
2.2.1.1. Divergent Approach

In a divergent approach, the dendrimer is grown outwards from a multifunctional core. The functional core molecule reacts with one reactive group in the building block (multifunctional monomer) in which other functional groups are protected for further reactions. The peripheral protective groups are then deprotected, ready for reaction with the active functional group of the next protected monomer unit. The process of protecting and deprotecting the peripheral functional groups is repeated to yield the increasing generations (Figure 2.4). However, in the divergent methods, side reactions cause structural defects, making it difficult to produce higher generations with precise structure.

Figure 2.4. The divergent approach.

Tomalia at al. (1985) developed branched poly(amidoamine) (PAMAM) using a divergent approach. As the cascade synthesis, the synthetic route involves Michael addition of ammonia to methyl acrylate, followed by amidation of the ester groups with ethylene diamine (Figure 2.5). The resulting primary amine groups then were reacted with methyl acrylate, followed by ethylene diamine. Repetition of the reaction sequence led to dendrimers of up to tenth generation.
Arborol, reported by Newkome et al. (1985), was also synthesized by a divergent synthetic route. The first step is a treatment of 1,3,5-tris(bromomethyl)benzene with NaC(CO₂Et)₃. The next step is the reaction of the ethyl ester groups with trisamine in the presence of LiAlH₄. This leads to hydroxyl functional groups (Figure 2.6). The highly branched arborol system is water-soluble with hydroxyl terminal groups. The higher generation growth was achieved by the repetition of above sequence.

The convergent approaches were developed in order to minimize the weaknesses of the divergent methods. This strategy proceeds in the opposite direction, from the periphery to the core by linking dendrons to the reactive core unit (Figure 2.7). The convergent approaches produce dendrimers that are relatively easy to purify and have...
minimal defects. However, the convergent methods do not easily yield high generations because of steric hindrance when doubling up the dendrimer size at each coupling step.

Figure 2.7. The convergent approach.

Fréchet and Hawker described the synthesis of aryl ether dendritic macromolecules as the first example for the convergent approach. The repetitive two-stage synthesis of aryl ether dendrons is based on the reaction of a 1,3,5-trihydroxybenzyl alcohol with benzyl bromide. The second step involves the transformation of the dendronized benzyl alcohol groups into benzyl bromide groups in the presence of CBr$_4$/PPh$_3$. Repetition of the two step process leads to successive generations up to the sixth generation bromide [G6]-Br. The dendritic macromolecules were prepared by attaching the dendrons to 1,1,1-tris(4’-hydroxyphenyl)ethane as a polyfunctional core. For example, the conjugation of forth generation [G4]-Br with the core gave the tri-alkylated dendritic macromolecules (Figure 2.8).
Percec et al. designed supramolecular dendrimers by self-assembly of dendrons, which were prepared by the convergent approach using the methyl 3,4,5-trishydroxybenzoate AB$_3$ building block (Figure 2.9). The first step is the reaction of 3,4,5-tris(n-dodecan-1-yloxy)benzoate with LiAlH$_4$, followed by chlorination with SOCl$_2$. The methyl 3,4,5-tris[3,4,5-tris(n-dodecan-1-yloxy)benzyloxy)] benzoate dendron was obtained by the alkylation of methyl 3,4,5-trihydroxybenzoate with the chlorinated intermediate. The repetitive cycles were applied to yield generation 4 dendrons, which were able to assemble into spherical micellar structures.
Newkome and coworkers reported the synthesis of a triethylene glycol (TriEG)-based dendrimer via a convergent approach (Figure 2.10). The TriEG dendron was prepared by the esterification of an α-azido-ο-carboxyl TriEG with a triester amine using the activated ester method (DCC; N,N'-dicyclohexylcarbodiimide). The TriEG dendrons were finally linked to a tetraacyl chloride core to generate the TriEG dendrimer structure (MALDI-ToF mass: m/z 8406.1, < 50% efficiency).
Because of their multifunctionality and water solubility, dendrimers quickly gained attention in biomedical applications.
2.3. Dendrimers in Biomedical Applications

2.3.1. PAMAM Dendrimers

Poly(amidoamine) PAMAM dendrimers, also known as “starburst dendrimers”, were commercialized first. \(^67,72\) The modified PAMAM synthesis is initiated using an alkyldiamine core, followed by Michael addition onto methyl acrylate to produce a branched intermediate. Amidation of the ester groups with excess ethylene diamine completes the cycle (Figure 2.11). Synthesis of higher generations of dendrimers could be achieved by sequentially repetitive reaction steps of Michael addition and amidation. However, the dendrimer growth eventually reaches a critical point where the steric crowding of the branching arms limits their extension into higher generations and produces defective branching architectures. \(^72\) Currently G0~G10 dendrimers with 500 ~ 1,000,000 g/mol are commercially available from Aldrich (Dendritech\textsuperscript{®}, Michigan, USA).

![Figure 2.11. Modified synthesis of PAMAM dendrimers.](image)
In addition to the structural control at the nanoscale size observed with dendrimers, another outstanding feature is their mimicry of globular proteins. Based on their systematic, dimensional length scaling (Figure 2.12), electrophoretic\textsuperscript{73} and other biomimetic properties,\textsuperscript{74,75} they are often referred to as ‘artificial proteins’.\textsuperscript{72} Within the PAMAM dendrimer family, the sizes of different generations match the sizes of many important proteins and bioassemblies. For example, insulin (≈30 Å), cytochrome C (≈40 Å) and hemoglobin (≈55 Å) are approximately the same size and shape as G3, G4 and G5 PAMAM dendrimers (Figure 2.12).

![Figure 2.12. Size comparison between (top) proteins and (bottom) PAMAM dendrimers.\textsuperscript{72,76}](image)

Unfortunately, Tomalia’s method for producing dendrimers is susceptible to defects, even at lower generations, thereby lowering yields, as well as destroying dendrimer symmetry (Figure 2.13). The primary cause of defects occurring during the synthesis is incomplete Michael addition, resulting in asymmetrical structure. To minimize the incomplete Michael addition, large excess of methyl acrylate is used in the
reaction. Fragmentation due to the retro-Michael additions is another cause of defects. Another potential problem is the complete removal of unreacted ethylenediamine (EDA) after the amidation occurs. If EDA is not removed completely, it can react with the next methyl acrylate increment during the iterative sequences and form new lower generation dendrimers, which can contaminate the desired dendrimer product. This type of impurity would become increasingly damaging to product quality as the reaction is continued over multiple generations. Intramolecular cyclization can occur during the amidation step. All of these structural defects decrease the purity of dendrimer. Because of this, the G5 PAMAM dendrimer has a distribution of structures, with an average of 128±20 –NH₂ groups in the outer corona.

Figure 2.13. Side reactions leading to structural defects during PAMAM dendrimer synthesis.

PAMAM is the most published dendrimer for biomedical applications.
2.3.2. PPI Dendrimers

The synthesis of poly(propylene imine) PPI dendrimers is based on the cascade reaction designed by Vögtle et al. in 1978 (Figure 2.3).\(^6\) Michael addition of a primary diamine to acrylonitrile yields a four-functional nitrile (Figure 2.14). The nitrile groups are then reduced to primary amine by Co(II)-borohydride complexes or diisobutylaluminum hydride. Subsequent cycles of Michael addition followed by reduction leads to the desired generation. In PPI dendrimers structural defects can occur through incomplete Michael addition, similarly to PAMAM dendrimers as described in section 2.3.1. PPI dendrimers have also been studied for drug delivery, gene transfection, imaging agents, nano-containers and nano-scaffolds.\(^7\)\(^6\)\(^7\)\(^8\)\(^7\)

![Figure 2.14. Synthesis of PPI dendrimers with an ethylene diamine core.](image)

2.3.3. Aliphatic Polyester Dendrimers

Aliphatic polyester dendrimers, which are considered biodegradable, are prepared by inclusion of ester groups in the main structure, which can be chemically hydrolyzed by
body fluids or enzymatically degraded by esterases in a biological environment. For example, Fréchet et al. synthesized oligoester dendrimers via both convergent and divergent approaches (Figure 2.15).\textsuperscript{89} The free HO-group of a double-protected triol was conjugated to a isopropylidene-protected carboxylic acid. The protected allyl carbonate was deprotected with Pd(PPh\textsubscript{3})\textsubscript{4} in the presence of morpholine to produce a free OH. The free hydroxyl group reacted with dibenzylidene-2,2-bis(oxymethyl)propionic anhydride to give the dendron. The sequential coupling and deprotection afforded the polyester dendrimer. Because of their biodegradability and biocompatibility, polyester dendrimers have been utilized for delivery of anticancer drugs,\textsuperscript{81} boron neutron capture agents,\textsuperscript{82,83} and genes\textsuperscript{84,85} for treatment of different cancers. Aliphatic polyesters dendrimers and dendrons based on 2,2-bis(methyloyl)propionic acid are commercially available for Polymer Factory (Sweden).

Figure 2.15. Synthesis of polyester dendrimers (Fréchet et al.).
2.3.4. Carbohydrate Dendrimers

Carbohydrates interact with specific receptors on the cell surface. Several research groups developed multivalent carbohydrate dendrimers to utilize the recognition and targeting of site-specific cells by accomplishing carbohydrate-receptor interactions. Roy and et al. first described the synthesis of a carbohydrate dendrimer. Triglycosylated trihydroxyamine was obtained in a multistep synthesis. The amino dendrons were then conjugated to the tricarboxylic acid core by dicyclohexycarbodiimide (DCC) coupling.

2.3.5. Amino Acid-based Dendrimers

Amino acid building blocks have unique properties such as chirality, amphiphilicity, biorecognition, and optical properties. Amino acid based dendrimers have been investigated to utilize the unique advantages of amino acid building blocks. Amino acid dendrimers have distinctive internal composition, which is able to provide stereoselective sites for non-covalent interactions with drug molecules. Specific structural folding in the branching units generates chiral dendrimers, which can be applied to drug targeting. Newkome et al. described one of the first amino acid functionalized dendrimers, which was synthesized by grafting tryptophane to the surface of poly(ether amide) dendrimers to modulate the encapsulation of hydrophobic molecules into the voids of the dendritic carrier.

2.3.6. PEG Dendrimers

Poly(ethylene glycol) PEG and their derivatives are widely known for their non-toxic nature, biocompatibility, and solubility in water and a wide range of solvents. PEG-
functionalized proteins and polymers were shown to find applications in the area of drug delivery systems.\textsuperscript{54,90} These PEGylated constructs can solubilize small hydrophobic drug molecules in water. Thus, PEG is one of most applicable materials in the design of biocompatible dendrimers for drug delivery systems.\textsuperscript{33,34,54,90-92} PEG is typically conjugated to the surface groups of a dendrimer to increase the solubility and shield the hydrophobic core.

Gnanou and et al. reported the synthesis of dendritic PEG structures prepared by an iterative divergent approach combining anionic ring-opening polymerization (AROP) of ethylene oxide from multi-hydroxylated precursors and subsequent branching of the PEG chain ends (Figure 2.16).\textsuperscript{93-96} PEG\textsubscript{G1(OH)}\textsubscript{3} (90\% conversion) was prepared by AROP of ethylene oxide with 1,1,1-tris(hydroxymethyl)ethane as an initiator, in the presence of diphenylmethylpotassium. The nucleophilic substitution of allyl chloride with PEG\textsubscript{G1(OH)}\textsubscript{3} followed by the oxidation of the allyl groups in the resulting polymer using OsO\textsubscript{4} and subsequent polymerization with ethylene oxide yielded PEG\textsubscript{G1(OH)}\textsubscript{6}. PEG dendrimers up to $M_n=900,000$ g/mol ($M_w/M_n =1.28$) with theoretically 384 outer hydroxy functional groups were obtained, but the overall efficiency was less than 20\%. The authors did not discuss the use of these dendrimers for biomedical application.
Hildgen and et al. reported the synthesis of PEG dendrimers with different end groups prepared by stepwise convergent approach using traditional condensation methods (Figure 2.17). First, the core was prepared by the esterification of butane tetracarboxylic acid with aspartic acid (90% conversion). Then the PEG dendron was synthesized convergently by the esterification of α-methacrylate ω-chloride PEG with 3,5-dihydroxybenzoic acid and gallic acid, followed by esterification with PEG. The final step was the conjugation of the core with the PEG dendrons to generate methacrylate-functionalized PEGs ($M_n=10,096$ g/mol). The overall efficiency was 50% with theoretically 24 outer methacrylate functional groups. The dendrimers demonstrated the ability to encapsulate rhodamine and β-carotene as hydrophilic and hydrophobic model compounds, respectively. The release of both rhodamine and β-carotene was found to be slow and sustained, with approximately 90% of drug released in 170 h. The authors
claimed that polymeric architectures with a PEO interior can potentially be used for the delivery of both hydrophobic and hydrophilic drugs.

Figure 2.17. Synthesis of methacrylate-functionalized PEG dendrimer by Hildgen and coworkers (2006).
Astruc et al. reported the synthesis of triethylene glycol-terminated PEG (G0-G2) dendrimers prepared by click chemistry to stabilize gold nanoparticles (Figure 2.18). A nona-azide core was prepared by CpFe+-induced nanoallylation of mesitylene in the presence of KOH and allyl bromide, followed by visible-light photolysis in acetonitrile in the presence of PPh$_3$ to remove the CpFe$^+$ group. Hydrosilylation with HSiMe$_2$CH$_2$Cl and Karsted catalyst reaction with NaN$_3$ yielded the multifunctional core. Williamson reaction between the nona-chloromethyl core and a Percec-type dendron were applied to prepare the PEG dendrons. Finally, the PEG dendrons were linked to the core using the Cu$^+$-catalyzed click reaction. The PEG dendrimers were used to encapsulate and stabilized gold nanoparticles.

2.4. Biocompatibility of Dendrimers

In order to be used in biomedical applications, dendrimers must be biocompatible; i.e., non-toxic, non-immunogenic and biopermeable (able to cross
biobarriers). They also should be able to stay in circulation in the biological system for the desired clinical effect.

Table 2.2. summarizes information about the biocompatibility of various dendrimers. Biological properties are extensively affected by the size of the dendrimers and by the surface functional groups on the specific dendrimers. The inner dendritic environments are of less importance; however, an aromatic interior may cause haemolysis via hydrophobic membrane contact.

Dendrimers with cationic surface groups: Dendrimers or other molecules with cationic surface groups cause destabilization of the cell membrane and show cytotoxicity. The cationic molecules are adhered to the negative cell surface groups by electrostatic attraction, followed by either hole formation or endocytosis. The primary amine surface groups of cationic dendrimers seem to be the main reason for cytotoxicity. The degree of substitution of the surface amine groups also affects the cytotoxicity of dendrimers. Primary amines are more toxic than secondary and tertiary alkyl amines. By alkylation or acylation of the charged surface amines, the cytotoxicity of dendrimers can be decreased by shielding the basic nitrogen atoms (Figure 2.17). The cytotoxicity of PAMAM dendrimers was found to be strongly dependent on the number of generations, with the higher generations being the most cytotoxic. Zinselmeyer and co-workers found generation dependent cytotoxicity in PPI dendrimers as well.

\[ G2-PPI > G1-PPI > G3-PPI > G4-PPI > G5-PPI \]

\[ \leftarrow \text{Low toxicity} \quad \rightarrow \text{High toxicity} \]

The generation-dependent cytotoxicity is in good agreement with the cytotoxicity of polymers which is proportional to their molecular size.
Table 2.2. Summary of biocompatibility of dendrimers with various surface groups.

<table>
<thead>
<tr>
<th>Surface Group</th>
<th>Chemical structure</th>
<th>Dendrimer</th>
<th>Biocompatibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cationic surface</td>
<td><img src="image1" alt="Chemical structure" /></td>
<td>PAMAM PEI PPI</td>
<td>a In vitro toxicity: + ( \text{IC}_{50} ): concentration at which 50% inhibition of mitochondrial dehydrogenase activity is measured. [ \text{in vivo toxicity: +}[ Cell uptake: + ]</td>
</tr>
<tr>
<td>Anionic surface</td>
<td><img src="image2" alt="Chemical structure" /></td>
<td>Lipid</td>
<td>In vitro toxicity: - [ Cell uptake: -/+ ]</td>
</tr>
<tr>
<td>Non-polar surface</td>
<td><img src="image3" alt="Chemical structure" /></td>
<td>Polyester Polyol Acetylated Polyol Ester-ether PEGylated</td>
<td>In vitro toxicity: - [ Cell uptake: - ]</td>
</tr>
<tr>
<td>Polar surface</td>
<td><img src="image4" alt="Chemical structure" /></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

+ ; positive response, - ; negative response

By covering surface amine groups with anionic groups or neutral groups such as acetyl and PEG (Figure 2.19), the cytotoxicity of dendrimers is abruptly lowered:

\[
\text{NH}_3^+ > \text{Guanidyl}^+ > \text{SO}_3^- > \text{PO}_3^- > \text{COO}^- > \text{PEG (-OH, -CH}_3\text{)}
\]
Introduction of PEG chains on the dendrimer surface groups leads to very low cytotoxicity. PEG chains suppress hydrophobic or ionic interactions with the cell membrane.

![Detoxification of primary amines by alkylation, ion-pairing or acylation.](image)

**Figure 2.19.** Detoxification of primary amines by alkylation, ion-pairing or acylation.

*Dendrimers with anionic surface groups:* Comparative toxicity studies depending on the different surface groups of amino-terminated and carboxyl-functionalized PAMAM dendrimers using Caco-2 cells (human colon carcinoma) show that the cationically charged PAMAM dendrimers have a significantly higher cytotoxicity than carboxyl functionalized ‘half generation’ PAMAM dendrimers.\(^{107}\)

*Dendrimers with non-polar or polar surface groups:* Cationic or anionic surface groups can be converted to neutral surfaces by lipid conjugation or PEGylation with significant reduction in cytotoxicity. Upon partial modification of the PAMAM dendrimer surface amines with chemically inert functionalities like PEG or fatty acids the cytotoxicity towards Caco-2 cells was reduced significantly (from IC\(_{50}\) ~ 0.13 mM to > 1 mM). It was found that partial modification with lipids or PEGs lowered the cytotoxicity. It was also found that modification of amino-terminated PAMAM dendrimers with PEG chains reduces immunogenicity and gives longer lifetime in the blood stream.\(^{108}\) Lipid-
modified polylysine dendrimers showed that these lipid modified dendrimers had poorer cell uptake.\textsuperscript{109}

2.5. Polymeric Conjugates for Cancer Treatment

The use of macromolecular engineering can produce polymeric structures for attachment of various bioactive molecules for targeting, imaging and detecting the cancer cells. As discussed in the Introduction, generation 5 poly(amidoamine) (PAMAM) dendrimers with 128 –NH\textsubscript{2} groups in the outer corona conjugated to an average of ~5 folic acid (FA) targeting ligands and ~5 methotrexate (MTX) drug molecules\textsuperscript{18,19}, have an enhanced binding to KB tumor cells\textsuperscript{20}, and target KB cells preferentially in cell culture\textsuperscript{16,21} and in xenograft tumors in mice.\textsuperscript{22} This section will provide background information on targeting, imaging and drug conjugation.

2.5.1. Targeting

Selectively delivering drugs to pathologic cells, thereby excluding the collateral damage that accompanies the drug intake by healthy cells\textsuperscript{9,110-112}, is a very important objective. Efforts to reach this goal included searching for ligands that bind selectively to pathologic cells, but displaying very low or no affinity for healthy cells. After identifying an optimal targeting ligand, linkers that would carry the conjugated drug to the pathologic cells with receptors for the selected ligand need to be constructed.\textsuperscript{113}
2.5.1.1. Vitamin-mediated Targeting

All living cells require vitamins for survival. Cancers which present rapidly dividing cells have great demand for certain vitamins by the over-expressed receptors in order to uptake the vitamins. Accordingly, agents conjugated to certain types of vitamins should be greatly attractive to cancer cells and useful in selective delivery of bioactive agents such as cancer drugs, dye and contrast agents. Recently, essential vitamins including vitamin B₁₂ (Cobalamin), folic acid (FA), biotin and riboflavin essential for cell division and growth have been spotlighted as targeting molecules (Figure 2.20).

![Image of vitamins: Folic acid, Biotin, Riboflavin, Cobalamin](image)

Figure 2.20. Various types of vitamins as targeting molecules.

The folate receptor, which is a glycosyl-phosphotidyl-inositol-anchored protein, exists in four major forms, namely FR-α, FR-β, FR-γ, and FR-δ forms.¹¹⁴,¹¹⁵ FR-γ and FR-δ are rarely detected in human tissues, while FR-α and FR-β are overexpressed in several malignant tumors. After the discovery of nondestructive folate
receptor-mediated endocytosis on the upregulated folate receptor (FR) of certain mammalian cell lines, the targeted delivery of FA conjugated with BSA, ribonuclease, horseradish peroxidase, IgG, and ferritin was first reported in 1991. Since then, FA has been recognized as an effective targeting agent for cancer tissues including ovarian, lung, breast, kidney, brain, endometrial, colon, and hematopoietic cell cancers that overexpress folate receptor proteins (FRPs) bound on the cell membrane. In contrast, in healthy cells, FRPs are underexpressed. Thus, direct conjugation of virtually any desired drug molecule to folate is a good strategy for targeting cancer cells and facilitating high tumor cell specificity of FA-linked drugs. These can be targeted with high binding affinity (K_d~10^-10 M). In addition, since FA and its drug conjugates enter cancer cells by receptor-mediated endocytosis, the normal permeability barriers that limit drug entry into cells can be overcome. This allows even very hydrophilic drugs / macromolecules to enter folate receptor-bearing cells as readily as their low molecular weight hydrophobic counterparts.

Three folate-conjugated chemotherapeutic agents are currently in human clinical trials (Figure 2.21). Basic design of those targeted molecules is the conjugation by a peptide spacer to increase water solubility together with disulfide linkages. The attached drug can be released by the reduction of disulfide linkage after receptor-mediated endocytosis due to the reducing environment inside the cells. EC145 and EC0489 with folate-conjugate desacetylvinblastine hydrazide as an immunotherapeutic drug were designed with different sizes of peptide spacers. EC0225 was designed as a dual-drug conjugate with folate.
Accordingly, the folate receptors were well recognized as potentially excellent biomarkers for targeted drug delivery and significant investigation has been made to date. In contrast, the other three vitamins have scarcely been investigated for tumor-targeting molecules until recently. Russell-Jones and coworkers investigated the update of vitamins into various tumor cell lines grown in vitro and in vivo.\textsuperscript{128} Folate, vitamin B\textsubscript{12} (Cbl) and biotin were conjugated to rhodamine-labeled lysine-modified hydroxypropylmethacrylic
acid to study the update in tumor cells. Table 2.3 summarizes the relative uptake of vitamin-targeted rhodamine-labeled polymer in various cell lines.\textsuperscript{128}

Table 2.3. Relative uptake of vitamin-targeted rhodamine-labeled polymers in various tumor cell Lines.\textsuperscript{128} Reprinted with permission from \textit{J. Inorg. Chem.} \textbf{2004}, 98, 1625-1633. Copyright © 2007 Elsevier.

<table>
<thead>
<tr>
<th>Tumour</th>
<th>Mouse</th>
<th>Type</th>
<th>Folate</th>
<th>Cbl</th>
<th>Biotin</th>
</tr>
</thead>
<tbody>
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<td>Balb/C</td>
<td>Bcell lymph</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>BW5147</td>
<td>AKR/J</td>
<td>Lymphoma</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>B16</td>
<td>C57/Bl</td>
<td>Melanoma</td>
<td>−</td>
<td></td>
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</tr>
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The folate-conjugated polymer showed enhanced uptake in leukemia and ovarian cancer cell lines. In contrast to the folate targeting, the vitamin B\textsubscript{12} (Cbl)-conjugated polymer had no uptake in leukemia and ovarian lines, but it showed increased uptake in colon, mastocytoma, lung, renal cell and breast tumor cell lines. The biotin-conjugated polymer showed the highest level of uptake: 5-25 times higher than the polymer only control.
Ojima and coworkers studied cell-specificity using a biotin-drug conjugate against three cell lines: L1210FR (biotin receptor overexpressed), L1210 (biotin receptors not overexpressed), and WI38 (normal human lung fibroblast, biotin receptor negative). The biotin-targeted taxoid with/without fluorescein (Figure 2.22) showed high specificity only to the biotin receptor overexpressed cell line (L1210FR).\textsuperscript{129}

![Figure 2.22. Chemical structure of biotin-S-S-taxoid and biotin-S-S-taxoid-fluorescein.](image)

2.5.1.2. Multivalent Targeting

Multivalent interactions (Figure 2.23), the simultaneous binding event of multiple ligands to multiple receptors in biological systems, have been extensively investigated to promote targeting of specific cell types.\textsuperscript{1,3,15,18,19,25,31,78} Such interactions are also central to a number of pathological\textsuperscript{65} processes including the attachment of viral, parasitic, mycoplasmal, and bacterial pathogens. The design of synthetic systems has been primarily approached through the development of molecular, multivalent inhibitors, which are stuck on the enzyme and prevents any substrate molecules from reacting with the enzyme.
Studies with biological multivalent inhibitors have yielded quantitative measurements of binding avidities on the order of 1 to 9 orders of magnitude. In contrast, synthetic nanoparticles needed for targeted drug delivery, have often exhibited much smaller improvements in binding avidities ranging from just 1 to 2 orders of magnitude improvement relative to single binding. Design and implementation of effective synthetic multivalent effector platforms capable of effectively targeting desired cell types \textit{in vivo} remains an important challenge. Furthermore, this is a challenge for which nanoscale polymer platforms are ideally suited since achieving multivalent interaction with multiple proteins or receptors requires the design of multivalent polymers that can simultaneously bind to surface regions of 10 to 100 square nanometers. Hong et al. reported the multivalent binding efficiency of FA-targeted PAMAM dendrimer for the folate binding protein (FBP). The success of these targeting experiments both \textit{in vitro} and \textit{in vivo} prompted detailed physical measurements to quantitatively test if the targeted polymers actually functioned via a multivalent
interaction. In order to test this hypothesis, a series of acetamide-terminated G5 dendrimer scaffolds containing ~2-14 folic acid molecules and AlexaFluor®488 dye (AF488) were synthesized (G5-Ac-AF488-FAₓ). Quantitative measurements of the association constant (𝐾_A) were obtained for the interaction of the devices with surface immobilized FBP using surface plasmon resonance (SPR, blue circles) and fluorescein-activated cell sorting (FACS, red squares). The nanodevice with an average of 2.6 FA showed a lower degree of cellular binding and association constant (𝐾_A) than the rest of the nanodevices. Remarkably, these effector systems exhibit a ~2,500–~170,000 fold enhancement of binding avidities as compared to free FA (Figure 2.24).

Figure 2.24. Comparison of the model study using SPR and the in vitro study using FACS of the effect of the number of FA per dendrimer molecule upon binding constant. Note that blue circles and red squares represent SPR and FACS results, respectively. The error bars represent standard deviations. The nanodevice with 2.6 FA shows a lower degree of cellular binding and association constant KA than the rest of the nanodevices. FACS data were obtained after incubation with dendritic nanodevices with FAR overexpressing KB cells at 37 C and represent averaging from 12 different samples at each condition. Association constants were averaged values from at least three SPR measurements for each point. The association constant (𝐾_A = 1/𝐾_D) is plotted in this case as it provides the best visual comparison to the FACS data.²³ Reprinted with permission from *Chem. Biol.* 2007, 14, 107-115. Copyright© 2007 Elsevier.
As previously discussed in Chapter I, even though FA-targeted PAMAM nanodevices showed great promise due to multivalent binding to cancer cells\textsuperscript{23}, the heterogeneity of the polymer itself led to the ultimate failure of this approach.\textsuperscript{31}

2.5.2. Imaging

Non-destructive techniques for visualizing cells have been used to detect and monitor tumor growth and other cancer activities. Fluorescein isothiocyanate (FITC) is a commonly used maker for optical imaging. FITC can be attached to the amine functional group of any macromolecules via a nucleophilic addition. FITC conjugated to a cancer cell targeted nanodevice can be selectively internalized by the cancer cells, allowing visualization of tumor sites.\textsuperscript{19} 6-Carboxytetramethylrhodamine (6-TAMRA) conjugated to dendrimer nanoparticles targeted by folic acid has also been used to visualize cancer cells \textit{in vivo} by its conjugation with.\textsuperscript{16} 6-TAMRA is more stable than FITC and it can be simply conjugated to the amine group. Similarly to FITC and 6-TAMRA, Alex Fluor\textsuperscript{®} 488 can also be used to visualize tumors.\textsuperscript{23}

![Chemical Structures]

Figure 2.25. Different types of imaging agents for visualizing cancer cells.
The biodistribution of radio-labeled folate-linked imaging agents ($^{111}$In-DTPA-folate and $^{99m}$Tc-DTPA-folate [EC20]) were clinically tested in humans (Figure 2.24). Uptake of $^{111}$In–DTPA–folate in ovarian cancer patients was seen in both the malignant tissues and kidneys, whereas only kidney uptake was observed in healthy individuals.$^{131}$ EC20, a $^{99m}$Tc-based folate-linked chelator (Phase II clinical trial) has proven to be the $\gamma$-emitter of choice for cancer and inflammation imaging due to its shorter half-life and its consequently lower radiation exposure.$^{132}$ EC17, a folate-fluorescein was tested in mouse liver. Picture A at the bottom of Figure 2.24 shows the normal white light images of the mouse liver, while picture C displays the fluorescent images of the same tissues.$^{133}$ Upon injection, the malignant tissues were visualized by the internalization of the folate conjugates (EC17).

Figure 2.26. Folate-conjugated imaging agents in human clinical trials.
The multivalent targeting principle could also be applied to increase the efficiency of *in vivo* imaging, provided that multifunctional biocompatible and water soluble polymers with precise structures would be available. Our group embarked on research towards the precision synthesis of multifunctional PEGs for this purpose.

2.6. New Approach for Synthesis of Multifunctional PEGs: Enzyme Catalysis

In tune with the globally increasing interest in “Green or Greener” chemistry, our group has been exploring the power of enzyme catalysis in the functionalization of synthetic polymers. Enzymatic catalysis in organic synthesis has emerged as an attractive “green chemistry” alternative to conventional chemical catalysis. In the past two decades, enzymatic catalysis has been applied to polymer synthesis and functionalization with several advantages, including high efficiency, recyclability, the ability to operate under mild conditions, and environmental friendliness.

All enzymes are classified into six main groups according to the International Union of Biochemistry and Molecular Biology (Figure 2.27). Today about 3000 enzymes are commercially available and some of them are mutated for industrial applications. Generally, oxidoreductases, hydrolases, and isomerases are relatively stable, and the most widely used catalysts in biotransformations. Some of isolated enzymes among them are conveniently used as catalysts in a practical manner. In contrast, lyases and ligases are present in lesser amounts in living cells and are less stable for isolation or separation from living organisms.
Lipases belong to the group of hydrolyses and are the most popular biocatalysts. They are widely used in esterification, transesterification, aminolysis, and Michael addition reactions in organic solvents.\textsuperscript{43} The most useful lipases for organic synthesis are: porcine pancreatic lipase (PPL), lipase from \textit{Pseudomoanas cepacia} (Amano lipase PS, PCL), lipase from \textit{Candida rugosa} (CRL), and lipase B from \textit{Candida antarctica} (CALB).\textsuperscript{140} 

CALB is one of the most effective catalysts in the resolution of alcohols and especially amines. CALB contains 317 amino acids and the catalytic triad is Ser105-His224-Asp187.\textsuperscript{141} It has a larger pocket above the Asp-His-Ser triad and a medium-sized pocket below it (Figure 2.28).\textsuperscript{142,143} The top pocket is the “carbonyl” pocket, and the bottom is the “hydroxyl” pocket, represented by different shading in Figure 2.28.
Comparison of the catalytic activities of CALB and distannoxane, a conventional tin-based catalyst revealed that the transesterification of vinyl acetate with 2-phenyl-1-propanol catalyzed by CALB was complete in 2 hours, while the traditional tin-based catalyst yielded 95% conversion in 12 hours (Figure 2.29).\(^{139}\)

![Figure 2.28. (Left) 3D structure of CALB [Image of PBD ID:1TCB (Uppenberg, J.; Hansen, M. T.; Patkar, S.; Jones, T. A., The sequence, crystal structure determination and refinement of two crystal forms of lipase B from *Candida antarctica*. *Structure* **1994**, 2 (4), 293-308) created with Polyview 3D] and (right) the catalytic triad of CALB.](image)

2.6.1. CALB-catalyzed Transesterification

Transesterification reactions are generally reversible. In order to change the reversible nature of the reaction into an irreversible type, the nucleophilicity of the leaving group of the acyl donor should be reduced by the introduction of electron-withdrawing groups such as trifluoroethyl- or trichloroethyl- into the ester. The use of enol esters such as vinyl or isopropenyl esters appears to be the most useful since they liberate unstable enols as by-products which rapidly tautomerize to give the corresponding aldehydes or ketones. Therefore, the reaction becomes completely irreversible. Acetaldehyde, which forms during the reactions with vinyl esters, is known to inactivate the lipases from Candida rugosa and Geotrichum candidum by forming a Schiff’s base with the lysine residues of the protein; however most lipases, including CALB, tolerate the liberated acetaldehyde. The catalytic cycle of the CALB-catalyzed transesterification of vinyl acrylate with tetraethylene glycol was visualized as shown in Figure 2.30, based on the mechanism from Bornscheuer and Kazlauskas. The different shadings represent the carbonyl and hydroxyl pockets of the enzyme.

First, the nucleophilic serine (Ser105) residue interacts with the carbonyl group of the vinyl acrylate, forming a tetrahedral intermediate which is stabilized by the oxyanion hole of the enzyme via three hydrogen bonds: one from glutamine (Gln106) and two from threonine (Thr40) units. In the second step, the ester bond is cleaved to form the first product, vinyl alcohol which will tautomerize to acetaldehyde, and the acyl-enzyme complex. In the third step, the reactant alcohol, tetraethylene glycol, reacts with the acyl-enzyme complex to form a second tetrahedral intermediate which is again stabilized by
the oxyanion hole. In the last step, the enzyme is deacylated to form the desired product. The nucleophilic attack by the Ser105 is mediated by the His224-Asp187 pair.

Figure 2.30. Illustration of the mechanism of CALB-catalyzed transesterification of vinyl acrylate with tetraethylene glycol. The different shading represents the two enzyme pockets.
2.6.2. CALB-catalyzed Michael Addition

Gotor et al. demonstrated CALB-catalyzed Michael-type addition of secondary amines to acrylonitriles and proposed that the serine in the active site was not involved in the reaction.\textsuperscript{147} He suggested that the reaction mechanism involved the activation of carbonyl group of the Michael-acceptor by the oxyanion hole, followed by histidine-aspartate-catalyzed proton transfer from the incoming nucleophile to the $\alpha$-carbon of the Michael-acceptor. According to the suggested mechanism,\textsuperscript{147,148} the catalytic cycle of the CALB-catalyzed Michael addition of diethanolamine to tetraethylene glycol diacrylate was visualized as shown in Figure 2.31. First the carbonyl group of tetraethylene glycol diacrylate is activated by the oxyanion hole of the enzyme. Then the conjugate addition of the incoming nucleophile, i.e. diethanolamine, to the tetraethylene glycol diacrylate takes place resulting in an intermediate which is stabilized by both the histidine-aspartate pair and the oxyanion hole in the enzyme active site. In the last step, the histidine-aspartate pair catalyzes the proton transfer from the diethanolamine to the $\alpha$-carbon of tetraethylene glycol diacrylate.
Figure 2.31. Illustration of the mechanism of CALB-catalyzed Michael addition of diethanolamine with tetraethylene glycol diacrylate.

The use of enzymes in polymer synthesis and post-polymerization functionalization has been reviewed.\textsuperscript{36,149}
2.6.3. Enzymes in the Synthesis of Telechelic Polymers

As discussed above, the synthesis of dendrimers involves telechelic reagents such as ethylene diamine. The use of telechelic polymers would be advantageous for increased flexibility of the dendrimer structure. Before our group had started working on the end-functionalization of preformed synthetic telechelic polymers, enzymatic catalysis has not been extensively utilized in the functionalization of preformed polymers despite the advantages enzymes offer. The first examples of quantitative functionalization of synthetic polymers using CALB-catalyzed reactions with and without organic solvents have been reported by our group.\textsuperscript{36-38} The primary hydroxyl groups of hydroxyl-functionalized polyisobutylene (PIBs) were quantitatively methacrylated by transesterification of vinyl methacrylate in the presence of CALB within 24 hours in hexane and 2 hours in bulk, respectively (Figure 2.32). Specifically, asymmetric methacrylation of α,ω-hydroxy-functionalized PIB was achieved by the regioselective transesterification of vinyl methacrylate using CALB in hexane within 24 hours, leaving the sterically hindered hydroxyl group intact.

![Diagram of CALB-catalyzed methacrylation of PIB-OHs](image)

Figure 2.32. CALB-catalyzed methacrylation of PIB-OHs. PIB-OH ($M_n=5200$ g/mol; $M_w/M_n=1.09$), Glissopal-OH ($M_n=3600$ g/mol; $M_w/M_n=1.34$), and asymmetric telechelic HO-PIB-OH ($M_n=7200$ g/mol; $M_w/M_n=1.04$).
Commercially available polydimethylsiloxanes (PDMSs): HO-PDMS-OH \((M_n = 3200 \text{ g/mol})\); PDMS-monocarbinol \((M_n = 5000 \text{ g/mol})\) and PDMS-dicarbinols \((M_n = 4500 \text{ g/mol and } 1000 \text{ g/mol})\) were transesterified with 1.5 eq. of vinyl methacrylate under solventless conditions within 2 hours in the presence of CALB (10 wt% relative to the total weight of reactants).\(^{36}\)

Primary hydroxy-functionalized polystyrene (PS-(CH\(_3\))\(_2\)Si-CH\(_2\)-OH, \(M_n = 2600 \text{ g/mol; } M_w/M_n = 1.06\))\(^{150}\) was quantitatively methacrylated by transesterification of vinyl methacrylate within 48 hours. In contrast, the ethylene oxide end-capped PS-OH did not react (Figure 2.33).

![Figure 2.33. Unsuccessful transesterification of vinyl methacrylate with PS-OH prepared by ethylene oxide end-capping (\(M_n = 2100 \text{ g/mol; } M_w/M_n = 1.07\)) and quantitative transesterification of vinyl methacrylate with PS-OH prepared by end-capping with (CH\(_3\))\(_2\)SiClH followed by hydrosilation with allyl alcohol (\(M_n = 2600 \text{ g/mol; } M_w/M_n = 1.06\)).](image)

PEGs with various molecular weights and molecular weight distributions were quantitatively functionalized with vinyl esters such as vinyl methacrylate and vinyl acrylate in THF in the presence of CALB within 24 hours (Figure 2.34).\(^{37}\)
Figure 2.34. CALB-catalyzed acetylation and methacrylation of PEGs.

Thymine-functionalized PEG was successfully prepared by the Amano lipase M-catalyzed Michael addition of thymine to PEG diacrylate within 72 hours. The PEG-diacrylate was prepared by the transesterification of vinyl acrylate with HO-PEG-OH ($M_n=2000$ g/mol, $M_w/M_n=1.91$) in the presence of CALB (Figure 2.35).

Figure 2.35. Preparation of thymine-functionalized PEG.

Recently, we were able to functionalize PEGs under solvent-free conditions within 4 hours, by dissolving low molecular weight HO-PEG-OH ($M_n=1000$ and 2000 g/mol) in the corresponding acyl donors at 50 °C. $^1$H and $^{13}$C NMR with MALDI-ToF confirmed quantitative conversion with the expected structures.

The synthesis of targeted drug carrier agents is very difficult, involving multistep processes. In addition, the solubility of these agents is very low in body fluids, hence of
the failure of several clinical trials. Effective conjugation of the mostly hydrophobic cancer drugs (see Appendix A) to water-soluble multifunctional polymers, including dendrimers, would be a great advance in the field and would allow the exploitation of the multivalent targeting concept. Thus we set out to design and construct multifunctional PEGs using enzyme catalysis.

2.7. Design for PEG-based Multivalent Targeting

As previously mentioned in Chapter I, the cancer targeted nanodevice based on G5 PAMAM dendrimers conjugated to an average ~ 5 folic acid (FA) targeting ligands and ~ 5 methotrexate (MTX) drug molecules\textsuperscript{18,19} demonstrated an enhanced binding to KB tumor cells\textsuperscript{20} and targeted KB cells preferentially in \textit{in vitro} and \textit{in vivo} studies.\textsuperscript{16,21,22} However, the multivalent PAMAM dendrimer samples used for the studies contain two major basic design flaws:

1. The polymer has a distribution of structures, with an average of 128±20 –NH\textsubscript{2} groups in the outer corona;

2. The targeting ligands are conjugated to the dendrimer in a stochastic fashion, generating a broad distribution of the ligands conjugated per dendrimer.

For example, HPLC analysis of a dendrimer sample with ~2.7 FA measured by \textsuperscript{1}H NMR showed that 16% had no targeting ligands, and 22% contained only 1 targeting ligand. Thus ~40% of the sample would have no multivalent binding. Only 13% of the sample actually had 3 FA molecules, and the rest had more than 3 ligands.\textsuperscript{31,151}
This thesis is aimed to address these flaws by the synthesis of poly(ethylene glycol) PEG star macromolecules with precisely controlled numbers of arms containing FA as a targeting ligand and/or fluorescein isothiocyanate (FITC) as an imaging agent. PEG was chosen because it shares many of the beneficial properties of PAMAM dendrimers, namely water solubility and resistance to recognition by the immune system.

Square topology was chosen as an initial starting point. PEG arm-length and topology were optimized by Brownian Dynamics simulations, performed by Professor Ronald Larson’s research group at the University of Michigan (Unpublished data). Brownian Dynamics provides a method for exploring the roles of macromolecular topology, arm-length, and receptor organization in determining the effectiveness of multivalent binding. Four-arm star polymers were modeled as 5 beads, one for the core and one for each of the arm ends (Figure 2.36).

![Figure 2.36](image)

Figure 2.36. The atomistic representation of the star polymer (left) may be approximated as 5 beads connected via spring with a known force vs. extension function (right).

The model assumed that the force of interaction between the core and each ligand is entropically dominated. This PEG extension model provided a linear force-extension relationship for short extensions and asymptotically increasing forces near the finite PEG. The polymer was simulated near a surface of receptors such that when a binding ligand is
in the proximity of the receptor and overcomes a barrier to binding, the targeting ligand binds to the receptor. Once bound, the polymer continues exploration of the molecular configurations which includes a bound polymer arm to the specific receptor with the possibility of additional arms binding (Figure 2.37). Over time, the polymer’s binding ligands may escape from the receptor and continue diffusing across the receptor surface. Simulation variables included (1) the polymer topology (number of arms), (2) the number of arms containing targeted ligands, (3) the arm length (4) the distribution of receptors on the surface, and (5) the association and dissociation rates of the receptors with individual ligands.

![Figure 2.37. Schematic illustration of a targeted star polymer binding to a surface containing four receptors.](image)

Preliminary data showed that the number of ligands per polymer that simultaneously bind to the surface is strongly dependent on polymer arm length (Figure 2.38). The experimentally determined association rate constant \((K_A = 25 \text{ M}^{-1}\text{s}^{-1})\) and dissociation rate constant \((K_D = 2\times10^{-5} \text{s}^{-1})\) for folic acid-folate binding protein and were employed as well as a receptor-receptor separation of 4 nm.\(^{23}\) For these parameters, an optimal number of repeat units per arm were calculated to be 20 for a square topology.
2.7.1. Synthetic Strategy

Based on the modeling described above, we designed the following synthetic pathway:

Step 1. Synthesis of a four-functional core by CALB-catalyzed transesterification of vinyl acrylate with TEG, followed by Michael addition of diethanolamine in the presence of CALB (Figure 2.39).
Step 2. Synthesis of vinyl-functionalized PEG arms. Vinyl ester-functionalized PEGs were to be prepared by the CALB-catalyzed transesterification of divinyl adipate (DVA). FA and FITC were to be attached to the arms.

Step 3. The functionalized arms subsequently were to be attached to the core via enzymatic transesterification (Figure 2.40).
We were hoping to get precise structures with the help of enzymatic catalysis. This approach will allow to compare devices with 1, 2 and 3 FA. A construct with 4 FA and FITC can be visualized by attaching the FITC into the middle of the core.
CHAPTER III
EXPERIMENTAL

3.1. Materials

Tetrahydrofuran (THF, bp: 66 °C, Fisher Scientific) and hexane (Hx, bp: 68 °C, Fisher Scientific) were distilled over sodium (≥ 99.9%, Aldrich) and benzophenone (99%, Aldrich) prior to use. Candida antarctica lipase B (CALB, 33273 Da, 20 wt% immobilized on a macroporous acrylic resin, Novozyme® 435, Sigma), ethyl acetate (≥ 99.5%, bp: 77 °C, Aldrich), vinyl methacrylate (98%, bp: 112 °C, TCI America), vinyl acrylate (> 90%, bp: 92 °C, Monomer-Polymer & Dajac Labs), vinyl crotonate (> 99%, TCI America), divinyl adipate (> 98%, TCI America), 2-hydroxyethyl acrylate (96%, Aldrich), 2-hydroxyethyl methacrylate (96%, Aldrich), dimethyl sulfoxide (anhydrous, ≥ 99.9%, Aldrich), N,N’-dimethylformamide (anhydrous, ≥ 99.8 %, Sigma-Aldrich), triethylamine (99%, Sigma-Aldrich), fluorescein isothiocyanate (Sigma-Aldrich), amine-functionalized polystyrene microbeads (0.8-1.4 mmol/g N loading, Sigma-Aldrich), mercaptoethanol (99%, Aldrich), 6-mercapto-1-hexanol (99 %, Aldrich), 11-mercapto-1-undecanol (97% Aldrich), tetraethylene glycol (99.5%, Aldrich), tetraethylene glycol monobenzyl ether (≥ 95%, TCI America), 12-bromo-1-dodecanol (≥ 98%, TCI America), folic acid (≥ 97%, Sigma), n-butyllithium (n-BuLi, 2.0 M in cyclohexane, Sigma), diethanolamine (≥ 99.5%, Aldrich), diethylamine (≥ 99.5%, Aldrich) and phosphomolybdic acid in 30% ethanol (TCI America) were used as received.
Poly(ethylene glycol) monomethyl ether with $M_n = 2000$ g/mol and poly(ethylene glycol) with $M_n = 2000$ g/mol having broad molecular weight distributions ($M_w/M_n$ value not provided) were purchased from Aldrich. Poly(ethylene glycol) monomethyl ether with $M_n = 750$ g/mol and poly(ethylene glycol) with $M_n = 2000$ g/mol having broad molecular weight distributions ($M_w/M_n$ value not provided) were purchased from Alfa Aesar. Poly(ethylene glycol) monomethyl ether ($M_n = 1100$ g/mol; $M_w/M_n = 1.09$), poly(ethylene glycol) monobenzyl ether ($M_n = 1000$ g/mol; $M_w/M_n = 1.01$ and $M_n = 2570$ g/mol; $M_w/M_n = 1.07$) and poly(ethylene glycol)s with $M_n = 1000$ g/mol ($M_w/M_n = 1.08$) were purchased from Polymer Sources Inc. $\alpha$-amino-$\omega$-hydroxy terminated poly(ethylene glycol) ($HO$-$\text{PEG}$-$\text{NH}_2$, $M_n=1000$ g/mol, $M_w/M_n=1.04$) was purchased from LaysanBio, Inc.

3.2. Procedures

3.2.1. Synthesis of the Four-functional Core [(HO)$_2$-TEG-(OH)$_2$]

3.2.1.1. Transesterification of Vinyl Acrylate with TEG in THF

Tetraethylene glycol (TEG, 0.50 g, 2.57 mmol, 0.44 mL, 1.01 mol/L) and vinyl acrylate (VA, 0.76 g, 7.75 mmol, 0.81 mL, 3.04 mol/L 1.5 eq. per OH in TEG) were dissolved in 1.3 mL of dried THF. CALB (76 mg resin @ 20 wt% enzyme, $4.6 \times 10^{-4}$ mmol, $1.8 \times 10^{-4}$ mol/L) was added into the solution. The reaction flask was sealed and purged with nitrogen. The mixture was stirred at 300 rpm for 24 hours at 50 °C. The progress of the reaction was monitored with TLC using ethyl acetate as an eluent and phosphomolybdic acid as a staining reagent. The CALB was filtered out using a 0.45µm PTFE syringe filter. The THF and excess VA were removed by a rotary evaporator. The
yellowish liquid product was dried in a vacuum oven at room temperature for 24 h (0.77 g yield, 100% conversion).

3.2.1.2. Michael Addition of Diethanolamine to TEG Diacrylate in THF

1.0 g of TEG diacrylate (3.31 mmol, 0.90 mL, 0.68 mol/L) and 0.70 g of diethanolamine (DEA, 6.66 mmol, 0.64 mL, 1.38 mol/L, 1.0 eq. per acrylate) were dissolved in 3.3 mL of dried THF. CALB (110 mg resin @ 20 wt% enzyme, 6.6 × 10⁻⁴ mmol, 1.4 × 10⁻⁴ mol/L) was added into the solution under nitrogen atmosphere. The reaction was stirred at 300 rpm for 4 hours at 50 °C. The CALB was filtered out using a syringe filter. The reaction was monitored by TLC using hexane/THF (1/1; vol/vol) and phosphomolybdic acid as the staining agent. The THF was removed by the rotary evaporator (1.71 g yield, 97.5% conversion).

3.2.1.3. Michael Addition of Diethanolamine to TEG Diacrylate in DMSO

2.0 g of TEG diacrylate (6.62 mmol, 1.82 mL, 1.03 mol/L) and 1.40 g of DEA (1.40 g, 13.32 mmol, 1.28 mL, 2.08 mol/L, 1.01 eq. per acrylate) were dissolved in 3.3 mL of anhydrous DMSO. CALB (220 mg resin @ 20 wt% enzyme, 1.3 × 10⁻³ mmol, 2.1 × 10⁻⁴ mol/L) was added into the solution under nitrogen atmosphere. The reaction was stirred at 300 rpm for 2 hours at 50 °C. The CALB was filtered out using a syringe filter. The reaction was monitored by TLC using hexane/THF (2/1; vol/vol) and phosphomolybdic acid as the staining agent. The DMSO was removed by a vacuum pump at 40 °C for 3 days (3.21 g yield, 94.6% conversion).
3.2.1.4. Synthesis of the Four-functional Core under Solventless Conditions

Acrylated TEG was also prepared by the transesterification of vinyl acrylate with TEG in liquid state under solventless conditions. TEG (1.00 g, 5.15 mmol, 0.89 mL, 1.45 mol/L) was added into VA (2.50 g, 25.48 mmol, 2.65 mL, 7.20 mol/L, 1.5 eq. per OH in TEG), in the presence of CALB (85 mg resin @ 20 wt% enzyme, 5.1 × 10^{-4} mmol, 1.4 × 10^{-4} mol/L). The mixture was sealed and purged with nitrogen. The reaction was processed at 300 rpm speed for 4 hours at 50 °C. The reaction was monitored by TLC using hexane/THF (1/1; vol/vol) and phosphomolybdic acid as the staining agent (1.55 g, 100% conversion).

DEA (7.65 g, 72.77 mmol, 6.97 mL, 4.59 mol/L) was added into 100 mL of flask containing TEG diacrylate (10.00 g, 33.07 mmol, 8.89 mL, 2.09 mol/L) and CALB (275 mg resin @ 20 wt% enzyme, 1.7 × 10^{-3} mmol, 1.0 × 10^{-4} mol/L). The flask was purged with nitrogen three times. The reaction was stirred for 1 hour at 40 °C or 50 °C. Silica gel TLC was used for monitoring the progress of reaction. TEG diacrylate and DEA were not miscible initially. After 10 min, the solution turned into transparent. The reaction was monitored by TLC using hexane/THF (1/1; vol/vol) and phosphomolybdic acid as the staining agent. The THF was removed by the rotary evaporator. To remove the enzyme from the reaction, 20 mL of dried THF was added into the reaction (the product was too viscous to filter out the enzyme). The light yellow product was concentrated using the rotary evaporator and dried using a vacuum pump (16.90 g, 100% conversion).
3.2.2. Synthesis of the Vinyl-functionalized PEG-FITC Arms

3.2.2.1. Conjugation of FITC with H\textsubscript{2}N-PEG-OH

H\textsubscript{2}N-PEG-OH (100 mg, 0.10 mmol, 0.033 mol/L, \(M\_n = 1000\) g/mol; \(M\_w/M\_n = 1.04\)) in 3 mL of anhydrous dimethylformamide (DMF) was reacted with fluorescein isothiocyanate (FITC, 50.2 mg, 0.13 mmol, 0.043 mol/L) in the presence of triethylamine (0.2 \(\mu\)L) under nitrogen and ambient atmosphere for 16 hours.\textsuperscript{153} To remove the excess of FITC from the product, 100 mg of amine-functionalized polystyrene microbeads (PS-NH\textsubscript{2}, 0.8-1.4 mmol/g N loading) was added and stirred for 4 hours at room temperature. FITC-PS beads were removed by the filtration and the product was precipitated in diethyl ether/hexane mixture. The precipitate was dried using a vacuum pump.

3.2.2.2. Transesterification of Divinyl Adipate with FITC-PEG-OH

Divinyl adipate (DVA, 0.86 g, 4.3 mmol, 0.82 mL, 2.36 mol/L) was reacted with 0.3 g of FITC-PEG-OH (0.3 g, 0.22 mmol, 0.12 mol/L) in 1 mL of dried THF in the presence of CALB (18 mg resin @ 20 wt% enzyme, 1.1 \(\times\) 10\(^{-4}\) mmol, 5.9 \(\times\) 10\(^{-5}\) mol/L) for 24 hours at 50 °C. The enzyme was removed by the filtration. The solution was precipitated in large excess of hexane and the product was dried using a vacuum pump (0.312g, 91% conversion).
3.2. 3. Synthesis of the PEG-FA Arms

3.2.3.1. Conjugation of FA with 6-Bromo-1-hexanol and 12-Bromo-1-dodecanol

1.96 g of folic acid (4.44 mmol, 0.09 mol/L) was reacted with 1 molar equivalent of \textit{n}-BuLi (2.2 mL, 4.4 mmol) in 49 mL of anhydrous DMSO. The reaction was performed in a closed reactor under a nitrogen atmosphere at room temperature. The progression of the reaction was indicated by the evolution of butane which bubbled out of the solution. After 30 minutes, 25 mL of the solution was extracted for characterization and precipitated in 250 mL of THF. Subsequently, 1 molar equivalent of 6-bromo-1-hexanol (0.41 g, 2.24 mmol) was added to the flask containing the remaining 2.27 mmol of lithiated FA in anhydrous DMSO (25 mL). The reaction was allowed to proceed for 24 hours at room temperature under a nitrogen atmosphere. The final product was precipitated in 250 mL THF. The precipitate was washed with hexane to remove possible unreacted 6-bromo-1-hexanol.

2.00 g of folic acid (4.53 mmol, 0.10 mol/L) was dissolved in 45.2 mL of anhydrous DMSO under nitrogen atmosphere. 2.3 mL of \textit{n}-BuLi (4.53 mmol, 1 eq.) was added dropwise into the FA solution using a dropping funnel. The solution was stirred for 30 minutes at 20°C. Subsequently 1.30 g of 12-bromo-1-dodecanol (4.53 mmol, 0.1 mol/L, 1 eq.) was added into the reaction mixture. The solution was stirred for 24 hours at room temperature. The dark yellow product was precipitated in 400 mL diethyl ether and washed with hexane and THF. Finally the brownish product was washed with water to remove free FA and LiBr. The solid product was dried in a vacuum oven for further analysis.
3.2.3.2. Transesterification of Divinyl Adipate with PEG Monobenzyl Ether

2.0 g of Poly(ethylene glycol) monobenzyl ether (BzPEG-OH, , 2.0 mmol, 0.13 mol/L, $M_n = 1000$ g/mol; $M_w/M_n = 1.01$) was reacted with DVA (15.85 g, 79.96 mmol, 15.10 mL, 5.30 mol/L, 40 eq. per OH in the BzPEG-OH) in the presence of CALB (332 mg resin @ 20 wt% enzyme, $2.0 \times 10^{-3}$ mmol, $1.3 \times 10^{-4}$ mol/L). The mixture was stirred for 4 hours at 50 °C under nitrogen. After the reaction, 5 mL of THF was added to the mixture and the CALB was filtered out using a 0.45 µm PTFE syringe filter. The excess of DVA was removed by precipitating the product in hexane and the precipitate was collected by adding THF and concentrated by a rotary evaporator. The white solid was dried in a vacuum oven at room temperature. Same reaction procedure was applied to higher molecular weight BzPEG-OH. 1.0 g of BzPEG-OH, (0.39 mmol, 0.13 mol/L, $M_n=2570$ g/mol; $M_w/M_n=1.07$) was reacted with DVA (3.09 g, 15.56 mmol, 2.94 mL, 5.29 mol/L, 40 eq. per OH in the BzPEG-OH) in the presence of CALB (65 mg resin @ 20 wt% enzyme, $3.9 \times 10^{-4}$ mmol, $1.3 \times 10^{-4}$ mol/L).

3.2.3.3. Transesterification of BzPEG Vinyl Adipate with 12-Bromo-1-dodecanol

0.61 g of BzPEG-DVA (0.51 mmol, 0.03 mol/L) was reacted with 12-bromo-1-dodecanol (0.14 g, 0.51 mmol, 1.0 eq., 0.03 mol/L) in the presence of CALB (86 mg resin @ 20 wt% enzyme, $5.2 \times 10^{-4}$ mmol, $3.0 \times 10^{-5}$ mmol) in 17 mL of dried THF. The reaction was stirred for 2 hours at 50 °C. The CALB was filtered out by a syringe filter. The white solid product was collected by precipitating in hexane. The product was dried in a vacuum oven.
3.2.3.4. Conjugation of FA with BzPEG-Br

0.08 g of FA (0.18 mmol, 1.5 eq., 0.06 mol/L) was dissolved in 3 mL of anhydrous DMSO under nitrogen. 0.09 mL of n-BuLi (0.19 mmol, 2 M in cyclohexane) was dropwisely added into the solution. The solution was stirred for 30 minutes at room temperature. 0.2 g of BzPEG-Br (0.13 mmol, 0.04 mol/L) in 1 mL of anhydrous DMSO was added in the solution using a dropping funnel under nitrogen. The solution was stirred for 24 hours at room temperature under ambient environment. The product was precipitated in cold diethyl ether and then washed with diethyl ether/hexane mixture. The brownish product was dried in a vacuum oven.

3.2.4. Fundamental Studies

3.2.4.1. Transesterification of Vinyl Methacrylate with 2-(Hydroxyethyl) Acrylate in THF

To investigate the reaction conditions for CALB-catalyzed transesterification, 2-(hydroxyethyl) acrylate (2HEA) was used as primary hydroxyl-bearing compound. For a solvent-free conditions, vinyl methacrylate (VMA, 0.58 g, 5.17 mmol, 0.62 mL, 6.31 mol/L) was added into a flask containing 2HEA (0.20 g, 1.72 mmol, 0.20 mL, 2.10 mol/L) and CALB (28.7 mg resin @ 20 wt% enzyme, 1.7 × 10⁻⁴ mmol, 2.0 × 10⁻⁴ mol/L, 1⁻⁴ equivalents relative to 2HEA) under an inert atmosphere. For a solution reaction using THF, the same condition with different concentrations of 2HEA in dried THF [0.86 mL (1.02 mol/L), 1.72 mL (0.68 mol/L) and 17.2 mL (0.10 mol/L)] were used for the reactions. To investigate the effect of enzyme concentration, VMA (1.45 g, 12.93 mmol, 1.55 mL, 2.004 mol/L) was added into a flask containing 2HEA (0.50 g, 4.31 mmol, 0.49
mL, 0.68 mol/L) in the presence of 4.3 mL dried THF. Different amounts of CALB [0.7 mg (4.2 × 10^{-6} mmol, 6.6 × 10^{-7} mol/L), 7.0 mg (4.2 × 10^{-5} mmol, 6.6 × 10^{-6} mol/L), 70 mg (4.2 × 10^{-4} mmol, 6.6 × 10^{-5} mol/L) and 700 mg (4.2 × 10^{-3} mmol, 6.6 × 10^{-4} mol/L)] under N₂. All of reactions were stirred at 50 °C and monitored with TLC using ethyl acetate as the eluent and phosphomolybdic acid as the staining agent. The conversion was calculated by ^1H NMR integration (solvent: DMSO-d₆).

3.2.4.2. Transesterification of Vinyl Methacrylate with 2-hydroxyethyl Acrylate under Solventless Conditions

To investigate the effect of enzyme concentration under solventless conditions, VMA (1.45 g, 12.93 mmol, 1.55 mL, 6.31 mol/L) was added into a flask containing 2HEA (0.50 g, 4.31 mmol, 0.49 mL, 2.10 mol/L) and CALB 2.0 × 10^{-6} mol/L [0.7 mg (4.2 × 10^{-6} mmol)], 2.0 × 10^{-5} mol/L [7.0 mg (4.2 × 10^{-5} mmol)], 2.0 × 10^{-4} mol/L [70 mg (4.2 × 10^{-4} mmol)] and 6.0 × 10^{-4} mol/L [210 mg (1.3 × 10^{-4} mmol)]. The reaction was carried out at 50 °C while stirring the reaction mixture at 300 rpm and monitored with TLC using ethyl acetate as the eluent and phosphomolybdic acid as the staining agent. The conversion was calculated by ^1H NMR integration.

3.2.4.3. Chemoselectivity in Enzyme-catalyzed Transesterification: Transesterification of Divinyl Adipate with 11-Mercapto-1-undecanol

11-Mercapto-1-undecanol (0.49 g, 2.40 mmol, 2.0 mol/L, 1.43 mol/L) in 1.2 mL of dried THF was added into a flask containing DVA (0.50 g, 2.52 mmol, 0.48 mL, 1.50 mol/L) and 50 mg of CALB (50 mg resin @ 20 wt% enzyme, 3.0 × 10^{-3} mmol, 1.8 × 10^{-4}
mol/L) under nitrogen. The mixture was stirred at 300 rpm for 90 min at 50°C. The reaction was monitored by TLC using hexane/THF (1/2, vol/vol) as the eluent phosphomolybdic acid as the staining reagent. The solid CALB was removed by filtration. The liquid product was concentrated by a rotary evaporator. Silica gel column chromatography was used to purify the compound using hexane and THF mixture.

3.2.4.4. Stereospecificity in Enzyme-catalyzed Michael Addition

3.2.4.4.1. Michael Addition of Diethylamine to 2-(Acryloyloxy)ethyl Methacrylate

0.2 g of 2-(Acryloyloxy)ethyl Methacrylate (1.15 mmol, 0.19 mL, 1.64 mol/L) were reacted with 0.36 g of diethylamine (3.45 mmol, 0.51 mL, 4.93 mol/L, 3eq.) by the CALB (18 mg resin @ 20 wt% enzyme, 1.1 × 10^{-4} mmol, 1.6 × 10^{-4} mol/L)-catalyzed Michael addition reaction. The reaction was stirred at 300 rpm for 30 min at 50 °C. After the reaction, the enzyme was filtered out using 0.45 µm PTFE syringe filter. The excess of diethylamine was removed by a vacuum pump.

3.2.4.4.2. Transesterification of Vinyl Crotonate with 2-hydroxyethyl Acrylate

Vinyl crotonate (1.45 g, 12.92 mmol, 1.54 mL, 6.36 mol/L, 3.0 eq. relative to 2HEA) was added into a flask containing 2HEA (0.50 g, 4.31 mmol, 0.49 mL, 2.12 mol/L) and CALB (72 mg resin @ 20wt% enzyme, 4.3 × 10^{-4} mmol, 2.1 × 10^{-4} mol/L) under an inert atmosphere. The mixtures were stirred at 300 rpm for 4 hours at 50 °C. After the reaction, the enzyme was filtered out using 0.45 µm PTFE syringe filter. The excess of vinyl crotonate was removed by a rotary evaporator.
3.2.4.3. Michael Addition of Diethylamine to 2-(Acryloyloxy)ethyl Crotonate

0.2 g of 2-(acryloyloxy)ethyl crotonate (0.2 g, 1.15 mmol, 0.19 mL, 2.02 mol/L) was reacted with 0.36 g of diethylamine (0.36 g, 3.45 mmol, 0.38 mL, 6.05 mol/L, 3eq.) by the CALB (18 mg resin @ 20wt% enzyme, 1.1 × 10^{-4} mmol, 1.9 × 10^{-4} mol/L)-catalyzed Michael addition reaction. The reaction was stirred at 300 rpm for 30 min at 50 °C. The after the reaction, the enzyme was filtered out using 0.45 μm PTFE syringe filter. The excess of diethylamine was removed by a vacuum pump.

3.2.4.4. Michael Addition of Diethanolamine to TEG Dimethacrylate

1.19 g of DEA (12.14 mmol, 1.08 mL, 2.20 mol/L) was added into 50 mL of flask containing TEG dimethacrylate (1.83 g, 5.52 mmol, 1.69 mL, 1.0 mol/L) and CALB (184 mg resin @ 20 wt% enzyme, 1.1 × 10^{-3} mmol, 2.0 × 10^{-4} mol/L) in the presence of 2.76 mL anhydrous DMSO under nitrogen atmosphere. The reaction was stirred at 300 rpm for 24 hours at 50 °C. The progress of reaction was monitored by TLC (hexane/THF: 1/1) and 1H NMR.

3.2.4.5. Vinyl-ester Functionalization of TEGs

3.2.4.5.1. Transesterification of Divinyl Adipate with TEG in THF

TEG (1.00 g, 5.15 mmol, 0.89 mL, 0.08 mol/L) was added into a flask containing DVA (3.06 g, 14.44 mmol, 2.91 mL, 3.0 eq. per OH in TEG, 2.26 mol/L) and CALB (86 mg resin @ 20 wt% enzyme, 5.2 × 10^{-4} mmol, 8.1 × 10^{-5} mol/L) in 2.6 mL of dried THF. The flask was sealed and purged with nitrogen. The mixture was stirred at 300 rpm for 40 min at 50 °C. TLC using hexane/THF (1/1;vol/vol) as the eluent and phosphomolybdic
acid as the staining reagent was used to monitor the progress of reaction. After the reaction, the CALB was filtered out using 0.45 µm PTFE syringe filter. The product was concentrated by a rotary evaporator. The excess of DVA was removed by silica gel column chromatography using hexane/THF (5/1; vol/vol) as the eluent.

3.2.4.5.2. Transesterification of DVA with TEGs under Solventless Conditions

Four TEGs (0.20 g, 1.03 mmol, 0.18 mL) were reacted with different molar equivalents of DVA [1.5 eq. (0.61 g, 3.09 mmol, 0.58 mL, 3.60 mol/L), 3.0 eq. (1.22 g, 6.18 mmol, 1.16 mL, 4.61 mol/L), 5.0 eq. (2.04 g, 10.29 mmol, 1.94 mL, 4.85 mol/L) and 10.0 eq. (4.08 g, 20.59 mmol, 3.98 mL, 4.95 mol/L) per OH in TEG] in the presence of each CALB (17 mg resin @ 20wt% enzyme, 1.0 × 10⁻⁴ mmol). The flasks were sealed and purged with nitrogen. The mixtures were stirred at 300 rpm for 1 hour at 50 °C. After the reaction, the enzyme was filtered out using 0.45 µm PTFE syringe filter. The excess of DVA was removed by silica gel column chromatography using hexane/THF (5/1, vol/vol) as the eluent.

3.2.4.6. Vinyl-ester Functionalization of PEGs under Solventless Conditions

3.2.4.6.1. The Effect of Divinyl Adipate Concentration on Coupling using PEG Diols

DVA (3.96 g, 20.0 mmol, 3.77 mL, 10.0 eq. per OH) was added to a sealed and argon-purged flask containing PEG (1.00 g, 1.0 mmol, \( M_n = 1000 \text{ g/mol; Mw/Mn} = 1.08 \)) and CALB (100 mg resin @ 20wt% enzyme, 6.0 × 10⁻⁴ mmol, 1.6 × 10⁻⁴ mol/L). The mixture was stirred at 300 rpm for 4 hours at 50 °C. After the reaction, 5 mL of THF was added to dilute the mixture and the solid CALB was removed by filtration. The product
was precipitated using 100 mL of hexane and the excess of DVA was removed by excess of hexane and diethyl ether. The white precipitate was dried in a vacuum oven for further analysis. The same procedure was also repeated with PEGs with 2000 g/mol to investigate the effect of molecular weight on polymer coupling.

3.2.4.6.2. The Effect of Divinyl Adipate Concentration on Coupling using PEG Monomethyl Ether

DVA [1.5 eq. (0.27 g, 1.37 mmol, 0.26 mL, 5.33 mol/L), 3.0 eq. (0.54 g, 2.73 mmol, 0.52 mL, 5.31 mol/L), 5.0 eq. (0.90 g, 4.55 mmol, 0.86 mL, 5.30 mol/L) and 10 eq. (1.80 g, 9.1 mmol, 1.71 mL, 5.31 mol/L)] was added to a sealed and argon-purged flask containing MPEG-OH (1.00 g, 0.91 mmol, $M_n = 1100$ g/mol; $M_w/M_n = 1.09$) and CALB (100 mg resin @ 20wt% enzyme, $6.0 \times 10^{-4}$ mmol). The mixture was stirred at 300 rpm for 4 hours at 50 °C. After the reaction, 5 mL of THF was added to the mixture and the CALB was removed by using a 0.45 µm PTFE syringe filter. The polymer was precipitated twice into hexane to remove the excess of DVA and dried in a vacuum oven at room temperature. The effect of DVA concentration on the extent of polymer coupling was investigated by reacting MPEG-OH ($M_n = 1100$ g/mol, $M_w/M_n = 1.09$) with 1.5, 3 and 10 equivalents of DVA. The same procedure was also repeated with MPEG-OHs with $M_n = 750$ g/mol and 2000 g/mol using 5.0 equivalent of DVA to investigate the effect of molecular weight on polymer coupling.
3.2.5. Enzymatic Functionalization of TEGs

3.2.5.1. Transesterification of Vinyl Methacrylate with Tetraethylene Glycol

TEG (1.00 g, 5.15 mmol, 0.89 mL, 1.85 mol/L) was added into VMA (1.76 g, 15.70 mmol, 1.89 mL, 5.65 mol/L, 1.5 eq. per OH in TEG), in the presence of CALB (85 mg resin @ 20 wt% enzyme, 5.1 × 10^{-4} mmol, 1.8 × 10^{-4} mol/L). The mixture was sealed and purged with nitrogen. The reaction was processed at 300 rpm speed for 4 hours at 50 °C. The progress of the reaction was monitored with TLC using hexane/THF (1/1; vol/vol) as the eluent and phosphomolybdic acid as the staining reagent. After the reaction, the excess VMA was removed by using a rotary evaporator. The product was dissolved in 10 mL of THF and the insoluble enzyme was removed by filtration. THF was removed by a rotary evaporator. The yellowish liquid products were dried in a vacuum oven at room temperature for further analysis (1.69 g yield, 100% conversion).

3.2.5.2. Transesterification of Vinyl Crotonate with TEG

The same reaction procedures were applied to prepare crotonated tetraethylene glycol. 2.000 g of TEG (10.29 mmol, 1.78 mL, 1.88 mol/L) was reacted with vinyl crotonate (3.46 g, 30.89 mmol, 3.68 mL, 5.66 mol/L, 1.5 eq. per OH in the TEG) in the presence of CALB (171 mg resin @ 20 wt% enzyme, 1.0 × 10^{-3} mmol, 1.9 × 10^{-4} mol/L) at 50 °C. The reaction was monitored by TLC using hexane/THF (1/1; vol/vol) as the eluent.
3.2.5.3. Transesterification of Vinyl Esters with TEG Monobenzyl Ether

Tetraethylene glycol monobenzyl ether (BzTEG-OH) was enzymatically functionalized by transesterification of vinyl methacrylate or vinyl crotonate under solventless conditions. BzTEG-OH (0.50 g, 1.76 mmol, 0.45 mL, 1.63 mol/L) was reacted with liquid vinyl methacrylate or vinyl crotonate (0.59 g, 5.28 mmol, 0.63 mL, 4.89 mol/L 3.0 eq. per OH in the BzTEG-OH) in the presence of CALB (29 mg, 1.7 × 10⁻⁴ mmol, 1.6 × 10⁻⁴ mol/L). The mixture was sealed and purged with nitrogen. The reaction was stirred at 300 rpm for 2 hours at 50 °C. The progress of the reaction was monitored with TLC using ethyl acetate/hexane (2/1, vol/vol) as the eluent and phosphomolybdic acid as the staining reagent. After the reaction, the excess vinyl esters were removed under vacuum and the enzyme was removed by filtration. The yellowish liquid products were dried in a vacuum oven at room temperature for further analysis.

3.2.5.4. Synthesis of (HO)₄-TEG-(OH)₄

3.2.5.4.1. Transesterification of Divinyl Adipate with 2-(Hydroxyethyl) Acrylate

DVA (2.00 g, 10.09 mmol, 1.90 mL, 1.90 mol/L) was dissolved in 5 mL of dried THF. CALB (200 mg resin @ 20 wt% enzyme, 1.2 × 10⁻³ mmol, 1.5 × 10⁻⁴ mol/L) and 2HEA (1.17 g, 10.09 mmol, 1.16 mL, 1.25 mol/L) were added into the solution under inert atmosphere. The mixture was stirred for 3 hours at 50 °C. The reaction was monitored by TLC using hexane/THF (3/1, vol/vol) as the eluent phosphomolybdic acid as the staining reagent. The solid CALB was removed by filtration. The liquid product was concentrated by a rotary evaporator. Silica gel column chromatography was used to
purify the compound using hexane and THF mixture. The liquid product was dried in a vacuum oven (2.10g, 77.1% conversion).

3.2.5.4.2. Transesterification of 2-(Acryloyloxy)ethyl Vinyl Adipate with (HO)$_2$-TEG-(OH)$_2$

Tetra-hydroxyl TEG ((HO)$_2$-TEG-(OH)$_2$, 0.50 g, 0.98 mmol, 0.2 mol/L) in 4.8 mL of dried THF was added into a flask containing 2-(Acryloyloxy) ethyl vinyl adipate (1.62 g, 5.85 mmol, 1.80 mol/L, 1.5 eq. per OH) and CALB (65 mg resin @ 20 wt% enzyme, 3.9 × 10$^{-4}$ mmol, 8.1 × 10$^{-5}$ mol/L). The mixture was sealed and purged with nitrogen. The reaction was monitored by TLC using ethyl acetate/hexane (1/1; vol/vol). The reaction was stirred at 300 rpm for 18 hours at 50 °C. The THF was removed by using a rotary evaporator. The product was dried in a vacuum oven (1.28g, 84.7% conversion).

3.2.5.4.3. Michael Addition of Diethanolamine to (Acrylate)$_2$-TEG-(Acrylate)$_2$

Tetra-acrylated TEG ((Acrylate)$_2$-TEG-(Acrylate)$_2$, 0.96 g, 0.66 mmol, 1.03 mol/L) in 0.33 mL of anhydrous DMSO was reacted with DEA (0.34 g, 3.19 mmol, 0.31 mL, 4.98 mol/L, 1.2 eq. per acrylate group) in the presence of CALB (11 mg resin @ 20wt% enzyme, 6.6 × 10$^{-5}$ mmol, 2.0 × 10$^{-4}$ mol/L). The mixture was stirred at 300 rpm for 1 hour at 40 °C. The enzymes were filtered out using PTFE syringe filter. The solution was precipitated in 100 mL of cold diethyl ether to remove DMSO. The brownish viscous product was collected using THF and dried in a vacuum pump.
3.2.6. Enzymatic Functionalization of PEGs

3.2.6.1. Transesterification of Vinyl Methacrylate with PEG

In methacrylation of PEG by a transesterification with an example of HO-PEG-OH \((M_n=1000 \text{ g/mol}; M_w/M_n=1.08)\), VMA (0.56 g, 4.99 mmol, 0.60 mL, 8.32 mol/L, 5.0 eq. per OH) was added into a flask containing PEG (0.50 g, 0.50 mmol, 0.83 mol/L) and CALB (50 mg resin @ 20wt% enzyme, 3.0 \times 10^{-4} \text{ mmol, 10 wt% relative to PEG, } 5.0 \times 10^{-4} \text{ mol/L}). The reaction mixture was purged with inert gas and stirred for 4 hours at 50 °C. After the reaction, the mixture was dispersed in 5 mL of dried THF and filtered by 0.45 µm PTFE syringe filter to remove the enzyme. The product was concentrated by removing the excess of vinyl methacrylate and THF using a rotary evaporator. The concentrate was precipitated in 100 mL of hexane, and then washed with hexane and diethyl ether. The precipitate was dissolved in 5 mL of dried THF and concentrated by the rotary evaporator, and then the white product was dried in a vacuum oven at room temperature for further analysis (0.56g, 100% conversion). PEG with \(M_n = 2000 \text{ g/mol (} M_w/M_n = 1.20\) was also enzymatically methacrylated.

3.2.6.2. Transesterification of Vinyl Crotonate with PEG

Crotonated PEG was prepared by following the same procedure described above. 0.41 g of PEG (0.41 mmol, 0.89 mol/L, \(M_n=1000 \text{ g/mol; } M_w/M_n=1.08\)) was added into a flask containing vinyl crotonate (0.43 g, 3.81 mmol, 0.46 mL, 8.28 mol/L, 5.0 eq. per OH in PEG) and CALB (42 mg resin at 20wt% enzyme, 2.5 \times 10^{-4} \text{ mmol, 10 wt% relative to PEG, } 5.5 \times 10^{-4} \text{ mol/L}) in inert atmosphere. The reaction mixture was purged with inert gas and stirred for 4 hours at 50 °C. After the reaction, the mixture was dispersed in 5 mL
of dried THF and filtered by 0.45 µm PTFE syringe filter to remove the enzyme. The product was concentrated by removing the excess of vinyl crotonate and THF using a rotary evaporator. The product was dried in a vacuum oven (0.46g, 100% conversion).

3.2.6.3. Transesterification of Vinyl Acrylate with PEG

VA (0.32 g, 3.241 mmol, 0.34 mL, 9.53 mol/L, 5 eq. per OH) was reacted with 0.32 g of PEG (0.32 mmol, 0.94 mol/L, \(M_n=1000\) g/mol \(M_w/M_n=1.08\)) in the presence of CALB (35 mg resin @ 20wt% enzyme, \(2.1 \times 10^{-4}\) mmol, \(6.2 \times 10^{-4}\) mol/L) for 4 hours at 50 °C. After the reaction, the mixture was dispersed in 5 mL of dried THF and filtered by 0.45 µm PTFE syringe filter to remove the enzyme. The product was concentrated by removing the excess of vinyl acrylate and THF using a rotary evaporator. The product was dried in a vacuum oven (0.36g, 100% conversion).

3.2.6.4. Synthesis of \((\text{HO})_2\text{-PEG-}(\text{OH})_2\)

PEG diacrylate (1.01 g, 0.86 mmol, 0.43 mol/L) in 1.9 mL of anhydrous DMSO was reacted with DEA (0.20 g, 1.90 mmol, 0.18 mL, 0.91 mol/L, 1.1 equivalent per acrylate group of PEG) in the presence of CALB (123 mg resin @ 20wt% enzyme, \(7.4 \times 10^{-4}\) mmol, \(3.6 \times 10^{-4}\) mol/L) under nitrogen. The reaction was stirred at 300 rpm for 24 hours at 50 °C. The CALB was removed using a 0.45 µm syringe filter. The product was precipitated in 200 mL of diethyl ether, and then washed with 100 mL of hexane. The white solid product was dried in a vacuum oven at room temperature (1.18g, 98.3% conversion).
3.3. Characterization of Products

Nuclear magnetic resonance (NMR) spectroscopy, size exclusion chromatography (SEC), matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS), electrospray ionization mass spectrometry (ESI MS), thin layer chromatography (TLC) and column chromatography were utilized in this research.

3.3.1. Nuclear Magnetic Resonance (NMR) Spectroscopy

$^1$H and $^{13}$C NMR spectra were recorded on Varian Mercury 300 or Varian NMRS 500 spectrometer using deuterated chloroform (Chemical Isotope Laboratories, 99.8% CDCl$_3$) or deuterated dimethyl sulfoxide (Chemical Isotope Laboratories, 99.9% DMSO-d$_6$) as solvent. The resonances of non-deuterated chloroform at δ = 7.27 ppm and δ = 77.23 ppm and that of non-deuterated dimethyl sulfoxide at δ = 2.5 ppm and δ = 39.51 ppm were used as internal references for the $^1$H and $^{13}$C NMR spectra, respectively. $^1$H NMR samples were prepared in 5 mm NMR tubes with 25-50 mg of polymer dissolved in ca. 0.7 mL of NMR solvent and the spectra were acquired after 16-64 transients with a relaxation time of 1-10 sec. $^{13}$C NMR samples were prepared in 5 mm NMR tubes with 100-150 mg of polymer dissolved in ca. 0.7 mL of NMR solvent and the spectra were acquired after ca. 1,000-20,000 transients with a relaxation time of 1 sec.

3.3.2. Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-ToF MS)

MALDI-ToF mass spectra were acquired on a Bruker Ultraflex-III ToF/ToF mass spectrometer (Bruker Daltonics, Inc., Billerica, MA) equipped with a Nd:YAG laser (355
nm) for verifying the purity and mass distribution of the products. All spectra were measured in positive reflector mode. The instrument was calibrated prior to each measurement with an external poly(methyl methacrylate) (PMMA) standard. Individual solutions in anhydrous THF (99.5%, Aldrich) of polymer (10 mg/mL), 1,8,9-trihydroxyanthracene (dithranol, 20 mg/mL, 97%, Alfa Aesar), and sodium trifluoroacetate (NaTFA, 10 mg/mL, 98%, Aldrich) were mixed in the ratio of polymer:matrix:cationizing salt (1:10:2), and 0.5 µL of the resulting mixture were deposited on microtiter plate wells (MTP 384-well ground steel plate). After evaporation of the solvent, the plate was inserted into the MALDI source. The spectra were obtained an acceleration voltage of 20 kV. The attenuation of the Nd:YAG laser was adjusted to minimize unwanted polymer fragmentation and to maximize the sensitivity.

3.3.3. Electrospray Ionization Mass Spectrometry (ESI-MS)

ESI mass spectra were acquired with a Bruker Daltonics Esquire-LC ion trap mass spectrometer for the identification of mass. The sample was dissolved in anhydrous THF (99.5%, Aldrich) at 1µg/µl and mixed with a 1µg/µl solution of sodium trifluoroacetate cationizing agent (98%, Aldrich) as a in THF in the ratio 100:1(sample:salt) (v/v). Experimental conditions: positive mode; drying gas, nitrogen (8 L/min); drying temperature, 300 °C; nebulizer gas, nitrogen (10 psi). The sodiated solutions were introduced into the ESI source by direct infusion using a syringe pump at a flow rate of 250 µl/h.
3.3.4. Thin Layer Chromatography (TLC)

Thin layer chromatography analyses were carried out by spotting and developing the samples on aluminium-backed silica gel plates or PET-supported aluminium oxide plates with fluorescent indicator (Aldrich) using various eluents. The plates were then analyzed using either phosphomolybdic acid staining reagent or UV light ($\lambda = 254 \text{ nm}$).

3.3.5. Column Chromatography

Silica gel column chromatography was employed for the purification of some of the products. The silica gel (Dynamic Adsorbents Inc., Silica Gel Classic Column 60A) was added to a beaker containing the appropriate eluent; and the resulting slurry was poured to a column which was half filled with the eluent. After all of the silica gel was added, the solvent was allowed to drain through the column until the solvent level was just above the surface of silica gel. The crude product which was dissolved in the minimum amount of solvent was loaded onto the column and the solution was allowed to drain until it was again just above the level of silica gel. The eluent solvent was then run through the column and fractions were analyzed by TLC. The fractions having the pure product were then combined and the solvent was removed under reduced pressure.
CHAPTER VI
RESULTS AND DISCUSSION

The objective of this research was to design and synthesize a multifunctional poly(ethylene glycol) (PEG) topology using enzymatic catalysis. For this purpose, a four-functional core and the functional PEG arms with fluorescein isothiocyanate (FITC) and folic acid (FA) were prepared using both traditional organic chemistry and enzyme-catalyzed reactions. To get a better understanding of enzymatic catalysis, fundamental studies were performed with functionalization of TEGs and PEGs.

4.1. Synthesis of Four-arm Star PEG

4.1.1. Synthesis of the Four-functional Core [(HO)₂-TEG-(OH)₂]

4.1.1.1 Transesterification of Vinyl Acrylate with TEG in THF

Vinyl acrylate (VA, 0.76 g, 7.75 mmol, 0.81 mL, 1.5 eq. per OH in TEG) was transesterified with tetraethylene glycol (TEG, 0.50 g, 2.57 mmol, 0.44 mL) using CALB (76 mg resin @ 20wt% enzyme, $4.6 \times 10^{-4}$ mmol) in dried THF (1.3 mL) (Figure 4.1). The progress of the reaction was monitored with TLC using ethyl acetate as an eluent and phosphomolybdic acid as a staining agent. After 4 hours, new spots at $R_f = 0.58$ and 0.78 were detected in addition to the TEG spot at $R_f=0.17$. After 24 hours there was only one spot at $R_f=0.78$. 

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Figure 4.1. Reaction scheme and TLC monitoring of the transesterification of VA with TEG in the presence of CALB. [VA] = 3.04 mol/L, [TEG] = 1.01 mol/L; [CALB] = 1.8 × 10^{-4} mol/L.

Figure 4.2 shows the $^1$H NMR spectrum of the transesterification product. The resonance (a) at δ=4.55 ppm, corresponding to the -OH protons of HO-TEG-OH disappeared and the peak of the methylene protons adjacent to the hydroxyl group shifted to δ=4.42 ppm (b) after the reaction (MW = 302.32 g/mol, 0.77 g yield, 100% conversion). New resonances attributed to the vinylidene [δ=6.18 ppm (f)] and vinyl [δ=6.32 ppm (e) and δ=5.97 ppm (e’)] protons appeared with the integration ratios of (b):e):(e’):(f) as 2:1:1:1.
The $^{13}$C NMR spectrum of the product also confirmed the structure of TEG diacrylate (Figure 4.3). The carbons connected to the hydroxyl group in the starting material at $\delta=59.47$ ppm shifted downfield to $\delta=63.44$ ppm (B) after the reaction and the carbon resonances of the acrylate group appeared at $\delta=165.44$ ppm (G), $\delta=131.58$ ppm (E) and $\delta=128.20$ ppm (F) corresponding to carbonyl carbon, $\alpha$-carbon and the vinyl carbon connected to the $\alpha$-carbon, respectively.
Figure 4.3. $^{13}$C NMR spectrum of the transesterification product of VA with TEG (solvent: DMSO-$d_6$).

The product was also analyzed by electrospray ionization mass spectrometry (ESI-MS) using sodium trifluoroacetate (NaTFA) as a cationizing agent. The ESI-MS confirmed the formation of a single product with $m/z$ 325.1 [M+Na$^+$], which is in good agreement with the calculated monoisotopic mass of the expected product: 325.22 Da [$4 \times 44.03$ (C$_2$H$_4$O repeat units) + 126.11 (C$_6$H$_6$O$_3$ end groups) + 22.99 (Na$^+$)] (Figure 4.4).
The reaction was efficient and yielded the desired product.

4.1.1.2. Michael Addition of Diethanolamine in THF

Michael addition of diethanolamine (DEA, 0.70 g, 6.66 mmol, 0.64 mL, 1 eq. per acrylate) onto TEG diacrylate (1.0 g, 3.31 mmol, 0.90 mL) was attempted in dried THF (3.3 mL) in the presence of CALB (110 mg resin @ 20wt% enzyme, 6.6 × 10^{-4} mmol). TLC was used to monitor the reaction with an eluent of the mixture of hexane/THF (1/1; vol/vol) and phosphomolybdic acid as the staining agent. The reaction was incomplete after 2 hours, as the spot at $R_f=0.58$ corresponding to the TEG diacrylate was still observed. At 4 hours of reaction time, the starting spot disappeared and there was only one spot at $R_f=0.02$ (Figure 4.5).
Figure 4.5. Reaction scheme and TLC monitoring of the CALB-catalyzed Michael addition of DEA to TEG diacrylate in THF. [DEA] = 1.38 mol/L, [TEG diacrylate] = 0.68 mol/L; [CALB] = 1.4 × 10^{-4} mol/L.

Figure 4.6 shows the NMR spectra of the product after 4 hours (1.71 g yield, 97.5% conversion). Even though the starting material on TLC was not observed, the NMR indicates the presence of traces of unreacted acrylate groups at δ=5.8~6.3 ppm. The triplet resonance at δ = 2.35 ppm (f) assigned to the protons next to the carbonyl, and the resonance at δ = 2.73 ppm (e) as a triplet represents the protons next to the amine group. In the $^{13}$C NMR spectrum, the carbons connected to oxygen and/or amine groups shifted to δ=59.25 ppm (H) and δ=56.26 ppm (I) and the carbon resonances of the acrylate group shifted upfield to δ=50.10 ppm (E) and δ= 32.17 ppm (F) after the reaction.
4.1.1.3. Michael Addition of DEA in DMSO

Previously, Dr. Sen demonstrated quantitative Michael addition of thymine to VA in DMSO in the presence of CALB.\textsuperscript{149} Thus, Michael addition was also performed in DMSO. DEA (1.40 g, 13.32 mmol, 1.28 mL, 1.01 eq. per acrylate) was reacted with TEG diacrylate (2.0 g, 6.62 mmol, 1.82 mL) in anhydrous DMSO (3.3 mL) in the presence of CALB (220 mg, 1.3 × 10^{-3} mmol). TLC was used to monitor the reaction with an eluent...
of the mixture of hexane/THF (2/1; vol/vol). After 2 hours, the spot at \( R_f = 0.58 \) corresponding to TEG diacrylate already disappeared and the Michael addition product was observed at \( R_f = 0.02 \) (Figure 4.7).

Figure 4.7. Reaction scheme and TLC monitoring of the CALB-catalyzed Michael addition of DEA to TEG diacrylate in DMSO. [DEA] = 2.08 mol/L, [TEG diacrylate] = 1.03 mol/L; [CALB] = 2.1 × 10^{-4} \text{ mol/L.}

![Reaction scheme and TLC monitoring](image)

Figure 4.8 shows NMR spectra of the Michael addition product of diethanolamine to TEG diacrylate after 2 h in DMSO-d_{6}. The peak corresponding to the protons of hydroxyl groups appeared at \( \delta=4.22 \) ppm (g) after the reaction. The resonances of the vinylidene [\( \delta=6.18 \) ppm] and vinyl [\( \delta=6.32 \) ppm and \( \delta=5.97 \) ppm] protons in the acrylate group in TEG diacrylate shifted to at \( \delta = 2.73 \) ppm (e) and \( \delta = 2.35 \) ppm (f), respectively. The relative intensities [(b):(c):(e):(f):(g)] 1:1:1:1:1 indicated quantitative functionalization. The \(^{13}\text{C} \) NMR spectrum of the Michael addition product also confirmed the structure. The carbons connected to the hydroxyl groups and the amine groups at \( \delta=59.67 \) ppm (H) and \( \delta=56.20 \) ppm (I) appeared after the reaction and the carbon
resonances of the acrylate group shifted upfield to $\delta = 50.02$ ppm (E) and $\delta = 32.22$ ppm (F), respectively.

Figure 4.8. NMR spectra of Michael addition of DEA to TEG diacrylate in DMSO: (top) $^1$H NMR spectrum and (bottom) $^{13}$C NMR spectrum (solvent: DMSO-d$_6$).

Thus the four-functional core was synthesized successfully. We are not sure why Michael addition proceeds better in DMSO.

4.1.1.4. Synthesis of the Four-functional Core under Solventless Conditions

We are striving to find “greener” conditions in polymer synthesis. In this spirit we attempted the transesterification and Michael addition in the absence of solvents.
VA (2.50 g, 25.48 mmol, 2.65 mL, 1.5 eq. per OH in TEG) was transesterified with TEG (1.00 g, 5.15 mmol, 0.89 mL) catalyzed by CALB (85 mg resin @ 20wt% enzyme, 5.1 × 10^{-4} mmol, 10^{-4} eq. relative to mol of TEG) for 4 hours (Figure 4.9). The progress of the reaction was monitored by TLC using hexane/THF (1/1; vol/vol) as the eluent and phosphomolybdic acid as the staining agent. After 4 hours the spot at R_f = 0.02 corresponding to TEG disappeared and only one spot at R_f = 0.58 was observed. ^1H & ^13C NMR spectra confirmed the structure of the product (Figure 4.10). The yield was 1.55 g with 100% conversion.

Figure 4.9. Reaction scheme and TLC monitoring of the CALB-catalyzed transesterification of VA with TEG under solventless conditions. [VA] = 7.20 mol/L, [TEG] = 1.45 mol/L; [CALB] = 1.4 × 10^{-4} mol/L.
Figure 4.10. NMR spectra of the transesterification product of VA with TEG under solventless conditions: (top) $^1$H NMR spectrum and (bottom) $^{13}$C NMR spectrum (solvent: DMSO-d$_6$).

$(\text{HO})_2\text{-TEG-(OH)}_2$ was prepared by the Michael addition of DEA (7.65 g, 72.77 mmol, 6.97 mL, 1.1 eq. per $\text{–CH=CH}_2$ in TEG diacrylate) to TEG diacrylate (10.00 g, 33.07 mmol, 8.89 mL) in the presence of CALB (275 mg resin @ 20wt% enzyme, 1.7 ×
10^{-3} \text{ mmol}) under solventless conditions (Figure 4.11). The reaction was attempted at 40 °C and 50 °C for 1 hour.

![Reaction scheme](image)

Figure 4.11. Reaction scheme and TLC monitoring of the CALB-catalyzed Michael addition of diethanolamine to TEG diacrylate under solventless conditions. [DEA] = 4.59 mol/L, [TEG diacrylate] = 2.09 mol/L; [CALB] = 1.0 \times 10^{-4} \text{ mol/L}.

The progress of reaction was monitored by TLC using hexane/THF (1/1; vol/vol) as the eluent mixture. The reaction was incomplete after 30 minutes, as a small spot at R_f=0.58 corresponding to TEG diacrylate was still observed. After 1 hour of reaction time, there was only one spot at R_f \approx 0. The \textsuperscript{1}H NMR spectra of the products were identical to Figure 4.8. However, when the reaction was performed at 50 °C, the \textsuperscript{13}C NMR spectrum showed the carbon resonance of ethanol group next to oxygen at \( \delta = 72.47 \text{ ppm} \) (X), which means that there was a cleavage at ester bonds generating free hydroxy groups. In contrast to the reaction at 50 °C, there was no side product at 40 °C (Figure 4.12)
Figure 4.12. $^{13}$C NMR spectra of Michael addition of DEA to TEG diacylate in solventless conditions at (top) 50 °C and (bottom) 40 °C reaction temperature (solvent: DMSO-d$_6$).
The ESI-MS confirmed the formation of the desired product with a peak at $m/z$ 535.2 [M+Na$^+$], which is in good agreement with this ion’s calculated monoisotopic mass.
of 535.30 Da \([4\times 44.03 \text{ (C}_2\text{H}_4\text{O repeat unit)} + 126.11 \text{ (C}_{14}\text{H}_{28}\text{N}_2\text{O}_7 \text{ end groups)} + 22.99 \text{ (Na}^+\text{)}] \) (Figure 4.13(a)). However, there were additional peaks in the lower molecular weight region. Tandem mass spectrometry (MS/MS) was applied to identify these peaks. MS/MS is used to produce structural information about a compound by fragmenting specific ions inside the mass spectrometer and identifying the resulting fragment ions. This information can then be pieced together to generate structural information regarding the original molecule. The peaks in the MS/MS spectrum (Figure 4.13(b)) were identified as fragmented species from the \((\text{HO})_2\text{-TEG-(OH)}_2\); the same peaks are also found in the ESI spectrum, confirming that they are fragments from the \((\text{HO})_2\text{-TEG-(OH)}_2\) single product and are not impurities or byproducts.

In conclusion, the four-functional core was also successfully prepared by the transesterification of VA and Michael addition of DEA in the presence of CALB under solventless conditions. NMR spectroscopy, ESI-MS and MS/MS confirmed the structure of the product.

4.1.2. Synthesis of the Vinyl-functionalized PEG Arms
4.1.2.1. Synthesis of the Vinyl-functionalized PEG-FITC Arms
4.1.2.1.1. Conjugation of FITC with \(\text{H}_2\text{N-PEG-OH}\)

\(\text{H}_2\text{N-PEG-OH} \text{ (0.1 g, 0.10 mmol, } M_n=1000 \text{ g/mol; } M_w/M_n=1.04) \) in anhydrous dimethylformamide (DMF, 3.0 mL) was reacted with fluorescein isothiocyanate (FITC, 50.2 mg, 0.13 mmol) in the presence of triethylamine (TEA, 0.2 \(\mu\text{L}\)) under nitrogen and dark environment for 16 hours.\(^{153}\) To remove the excess of FITC from the product, 100
mg of amine-functionalized polystyrene microbeads (PS-NH₂, 0.8-1.4 mmol/g N loading) was added and stirred for 4 hours at room temperature (Figure 4.14).

The red PS-NH-FITC beads were filtered using a 0.45 μm PTFE syringe filter. The dark yellow product was collected by precipitation in diethyl ether and hexane.

Figure 4.15 shows the ¹H and ¹³C NMR spectra of FITC starting material, verifying the structure. All proton and carbon resonances corresponded to the expected structure. Figure 4.16 displays the ¹H NMR spectra of H₂N-PEG-OH and the conjugated product.
The proton resonances corresponding to the FITC residue \[\delta = 10.09 \text{ ppm (m)}, \delta = 7.95 \text{ ppm (g)}, \delta = 7.74 \text{ ppm (h)}, \delta = 6.67 \text{ ppm (i)}, \delta = 6.59 \text{ ppm (l)} \text{ and } \delta = 6.57 \text{ ppm (k)}\] were observed at the expected positions. The proton resonance next to amine at \(\delta = 2.71\) ppm (a) shifted downfield to \(\delta = 3.50\) ppm, overlapping with the protons of the PEG repeat units. The protons from the residual DMF can be observed at \(\delta = 2.89\) ppm and \(\delta = 2.73\) ppm.
Figure 4.16. $^1$H NMR spectra of (bottom) H$_2$N-PEG-OH ($M_n = 1000$ g/mol, $M_w/M_n = 1.04$) and (top) FITC-PEG-OH in DMSO-d$_6$.

Figure 4.17 shows $^{13}$C NMR spectra of H$_2$N-PEG-OH and FITC-PEG-OH in DMSO-d$_6$. The carbons next to the amine group of H$_2$N-PEG-OH shifted from $\delta=40.75$ ppm (A) and $\delta=71.69$ ppm (B) to $\delta=45.68$ ppm (A’) and $\delta=66.96$ ppm (B’), respectively, indicating quantitative conversion. Also, the signal of the carbon next to the OH in the H$_2$N-PEG-OH at $\delta=60.17$ (F) did not shift, indicating that the OH group remained intact.
Figure 4.17. $^{13}$C NMR spectra of (bottom) H$_2$N-PEG-OH ($M_n = 1000$ g/mol; $M_w/M_n = 1.04$) and (top) FITC-PEG-OH in DMSO-d$_6$.

Furthermore, the carbon of the isothiocyanate group of FITC shifted from $\delta = 135.76$ ppm (G, Figure 4.15) to $\delta = 180.52$ ppm (G', Figure 4.17).
4.1.2.1.2. Transesterification of Divinyl Adipate with FITC-PEG-OH

Divinyl adipate (DVA, 0.86 g, 4.3 mmol, 0.82 mL, 20 eq. per OH) was reacted with FITC-PEG-OH (0.3 g, 0.22 mmol) in dried THF (1.0 mL) in the presence of CALB (18 mg resin @ 20wt% enzyme, $1.1 \times 10^{-4}$ mmol) for 24 hours (Figure 4.18).

![Figure 4.18. Transesterification of DVA with FITC-PEG-OH in dried THF in the presence of CALB. $[\text{DVA}] = 2.36 \text{ mol/L}, [\text{FITC-PEG-OH}] = 0.12 \text{ mol/L}; [\text{CALB}] = 5.9 \times 10^{-5} \text{ mol/L}$.](image)

The product was precipitated in diethyl ether and washed with hexane to remove the excess DVA. The yellowish precipitate (0.312 g, 91% conversion) was analyzed by $^1$H and $^{13}$C NMR spectroscopy. Figure 4.19 shows the NMR spectra of the product. The signal of the hydroxyl proton of FITC-PEG-OH at $\delta = 4.50$ ppm disappeared after the reaction and the signals assigned to methylene proton resonances next to the oxygen at $\delta = 4.12$ ppm (e) appeared. The protons of the vinyl ester group appeared at $\delta = 4.86$ ppm (r), $\delta = 4.63$ ppm (s) and $\delta = 7.18$ ppm (q). The relative integrals of the protons of the
newly formed vinyl adipate chain end [(q),(r),(s)] and the terminal methylene protons next to oxygen (q): (r): (s): (e) = 1: 1: 1: 2 demonstrate successful functionalization.

Figure 4.19. NMR spectra of the transesterification product of DVA with FITC-PEG-OH: (top) $^1$H NMR spectrum and (bottom) $^{13}$C NMR spectrum (solvent: DMSO-d$_6$).

The $^{13}$C NMR spectrum showed that the carbon connected to the hydroxyl group at $\delta=60.12$ ppm in the FITC-PEG-OH shifted downfield to $\delta=63.17$ ppm (F) and the carbon signals corresponding to the vinyl group appeared at $\delta=97.99$ ppm (V) and at
δ=141.18 ppm (U), respectively. The carbonyl carbons of the adipate appear at δ = 172.58 ppm (T) and δ = 170.17 ppm (Q).

In conclusion, vinyl ester functionalized FITC-PEG was successfully prepared by two steps of chemo-enzymatic reactions. FITC was conjugated to the amine group of H₂N-PEG-OH by nucleophilic addition to the isothiocyanate, forming a thiourea bond. Subsequently vinyl functionalized FITC-PEG was prepared by the transesterification of DVA with FITC-PEG-OH in THF in the presence of CALB.

4.1.2.2. Synthesis of the vinyl-functionalized PEG-FA arms

In most cases the conjugation of folic acid (FA) with drugs, imaging and bioactive agents is carried out by the so-called “activated ester” method using condensing agents such as N,N'-dicyclohexylcarbodiimide (DCC)⁸, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)⁹ and (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP)⁴ with or without activators such as N-hydroxy succinimide (NHS) and hydroxybenzotriazole (HOBt). However, since the carboxylic acid groups at the α and γ positions are in very similar chemical environment, their reactivity is very similar under the conditions normally used for conjugation. Consequently the regioselectivity toward the desired γ-conjugated product is quite poor and the product of these methods is a mixture of α-, γ, and αγ-conjugates so the γ functionalized FA needs to be isolated. Figure 4.20 shows the conjugation of FA with α-amino terminated poly(ethylene glycol) monomethyl ether
(MPEG-NH$_2$, $M_n=2000$ g/mol; $M_w/M_n = 1.11$) using PyBOP and $N,N$-diisopropylethylamine (DIPEA) in DMSO.

Figure 4.20. The activated ester approach for the conjugation of FA to MPEG-NH$_2$ using PyBOP and DIPEA in DMSO.

The magnified region of the $^1$H NMR spectrum (Figure 4.21) between $\delta=7.50$ ppm and $\delta=9.00$ ppm shows the doublet proton (r) resonance of the secondary amine in free FA at $\delta=8.09-8.12$ ppm, together with that of the $\gamma$- and $\alpha$-substituted FA (r' at $\delta=8.15-18$ ppm and r” at $\delta=7.92-7.95$ ppm) and the disubstituted FA (r”’ at $\delta=7.96-7.98$ ppm). The signals appear as doublets because of $J$-coupling. From the integration the
product contains ~40% γ-conjugated product with ~30% α- and disubstituted FA and ~30% free folic acid.

Figure 4.21. $^1$H NMR spectrum of MPEG-FA product using PyBOP (solvent: DMSO-d$_6$).

Exclusive γ-conjugation was achieved by a “retrosynthetic” approach.$^{161-164}$ This method is based on the cleavage of the FA group into pteroate and glutamate derivatives, followed by selective protection and reconstruction of the γ-FA. While this method leads
to exclusive γ-conjugation, it is a very tedious multistep process. For example, FA was cleaved into pteroic and glutamic acids using the carboxylpeptidase G hydrolase enzyme, followed by protection of the α-carboxylic acid site in the glutamic acid using 2-trimethylsilylethanol (TMSCH$_2$CH$_2$OH) and carbonyldiimidazole (CDI). The pteroate was also protected using a similar chemistry. The product was then reconstructed from the protected pteroate and glutamate moieties$^{164}$, yielding the FA with a free γ-site for exclusive conjugation with various bioactive molecules having an amino or a hydroxy group (Figure 4.22).
4.1.2.2.1. New Process for the γ-Conjugation of FA using Lithium Chemistry

We developed a new method based on lithium chemistry, shown in Figure 4.23. The first step involved the formation of the lithium salt of the γ-carboxyl group in the glutamic acid moiety of FA using 1 molar equivalent of an organometallic compound such as n-butyllithium. Subsequently the γ-lithiated FA is reacted with 1 equivalent of a compound that is able to react with the lithium salt effectively.

![Figure 4.23](image-url)

FA (1.96 g, 4.4 mmol, 0.09 mol/L) was reacted with 1 molar equivalent of n-BuLi (2.2 mL, 4.4 mmol, 2M in cyclohexane) in 49 mL of anhydrous DMSO. The reaction was performed in a closed reactor under a nitrogen atmosphere at room temperature. The progression of the reaction was indicated by the evolution of butane which bubbled out of the solution. After 30 minutes, 25 mL of the solution was extracted for characterization and precipitated in 250 mL of THF. Subsequently, 1 molar equivalent of 6-bromo-1-
hexanol (0.41 g, 2.26 mmol, 0.09 mol/L) was added to the flask containing the remaining 2.27 mmol of lithiated FA in anhydrous DMSO (25 mL). The reaction was allowed to proceed for 24 hours at room temperature under a nitrogen atmosphere. The final product was precipitated in 250 mL THF. The precipitate was washed with hexane to remove possible unreacted 6-bromo-1-hexanol. The $^{13}$C NMR spectra of the starting FA and the lithiated intermediate are shown in Figure 4.24. Upon lithiation, the peaks corresponding

Figure 4.24. $^{13}$C NMR spectra of (top) its lithiated intermediate and (bottom) FA (solvent: DMSO-d$_6$).
to the acid carbons (A) and (E) at $\delta=173.97$ and $\delta=173.78$ ppm shifted downfield to $\delta=175.58$ and $\delta=174.92$ ppm, and became more separated (0.66 ppm) as shown in the expanded regions of the $^{13}$C NMRs in Figure 4.25.

![Figure 4.25. Expanded region of $^{13}$C NMR spectra of its lithiated intermediate (top) and folic acid (bottom).](image)

The lithiated intermediate was then reacted with 6-bromo-1-hexanol. The $^1$H NMR spectrum of the product in Figure 4.26 shows the -CH$_2$- protons (x) of the conjugated hexanol group at $\delta=4.0$ ppm. There is no signal at $\delta=3.42$ ppm, characteristic of the CH$_2$ protons next to the Br group in 6-bromo-1-hexanol, indicating complete reaction. Figure 4.33 demonstrates the absence of the $\alpha$-folate and mixed product signals between 7.8 and 8.0 ppm, but shows the $\gamma$ –substituted NH proton signal ($r'$) at 8.2 ppm. The NH proton signal (r) at 8.05-8.06 ppm indicates the presence of residual free FA. The ratio of (u) and (x) to (b) and (c) is 0.9/1, indicating 90% conversion. The $m/z$ 564.2051 ([M+Na]$^+$) observed by ESI MS agreed very well with the calculated $m/z$ 564.5453.
Figure 4.26. $^1$H NMR spectra of (top) FA and (bottom) FA-hexanol in DMSO-d$_6$.

Figure 4.27 shows an expanded region of the $^{13}$C NMR spectrum of FA-hexanol. The disappearance of the peaks at 174.92 and 175.58 ppm indicate that the lithiated intermediate reacted completely. The small peak (173.97-173.99 ppm) near (E) indicates
the presence of some free FA, in agreement with the $^1$H NMR spectrum. This is most likely due to base-catalyzed hydrolysis during the water-wash of the Li-residues. The peak (E) at $\delta=173.95$ ppm is assigned to the carbon of the free $\alpha$-acid in a $\gamma$-substituted folate, based on the assignment by Mindt et al.\textsuperscript{163} The peak at $\delta=172.29$ ppm in Figure 4.27 is assigned to the substituted $\gamma$ carbonyl group; the substitution by an alkyl group is expected to shift the carbonyl signal upfield.

![Figure 4.27](image.png)

Figure 4.27. Expanded region of $^{13}$C NMR spectrum of the carbonyl carbons in FA-hexanol (solvent: DMSO-$d_6$).

The same reaction was carried out with 12-bromo-1-dodecanol, with similar results. FA (2.0 g, 4.53 mmol, 0.10 mol/L) was dissolved in 45.2 mL of anhydrous DMSO under nitrogen atmosphere. $n$-BuLi (2.3 mL, 4.53 mmol, 1 eq.) was added dropwise into the FA solution using a dropping funnel. The solution was stirred for 30 minutes at 20°C. Subsequently 1.30 g of 12-bromo-1-dodecanol (4.53 mmol, 0.1 mol/L) was added into the reaction mixture. The solution was stirred for 24 hours at room temperature. The dark yellow product was precipitated in 400 mL diethyl ether and
washed with hexane and THF. Finally the brownish product was washed with water to remove free FA and LiBr. The solid product was dried in a vacuum oven for further analysis.

The $^1$H NMR spectrum of FA-dodecanol in DMSO-d$_6$ is shown in Figure 4.28. There is no proton resonance signal at $\delta=3.45$ ppm, characteristic of the CH$_2$ protons next to the -Br group in 12-bromo-1-dodecanol. The CH$_2$ protons (x) of the dodecanol conjugated to the $\gamma$-carboxylic acid in FA appear at 4.00 ppm. The doublet signal (r') of the $\gamma$-substituted product appeared at $\delta=8.23-8.24$ ppm. The doublet (r) at $\delta=8.10-8.11$ ppm corresponds to the NH proton of some free FA. The ratio of (r') and (r) is 0.68/0.32, indicating 68% conversion. The m/z 648.4252 ([M+Na]$^+$) observed by ESI-MS agreed very well with the calculated m/z 648.7056.

Figure 4.28. $^1$H NMR spectrum of FA-dodecanol in DMSO-d$_6$. 
4.1.2.2. Transesterification of Divinyl Adipate with PEG Monobenzyl Ether

Benzyl protected PEG (BzPEG) was reacted with divinyl adipate (DVA) to have the vinyl ester functionality at the end of chain (Figure 4.29). The benzyl ether group can be removed by hydrogenation\textsuperscript{165, 166} and then the free alcohol can be conjugated with another reactive molecule. DVA (15.85 g, 79.96 mmol, 15.10 mL, 40 eq. per OH in the BzPEG\textsubscript{1000}) was transesterified with BzPEG\textsubscript{1000} (2.0 g, 2.0 mmol, \(M_n = 1000\) g/mol; \(M_w/M_n = 1.01\)) in the presence of CALB (332 mg resin @ 20wt% enzyme, \(2.0 \times 10^{-3}\) mmol).

Figure 4.29. CALB-catalyzed transesterification of DVA with BzPEG\textsubscript{1000} under solventless conditions. [DVA] = 5.30 mol/L, [BzPEG\textsubscript{1000}] = 0.13 mol/L; [CALB] = 1.3 \times 10^{-4}\) mol/L.

The \(^1\)H NMR spectra of the BzPEG\textsubscript{1000} and its transesterification product with DVA are shown in Figure 4.30. The hydroxyl protons of BzPEG\textsubscript{1000} at \(\delta = 4.53\) ppm (a) disappeared after the reaction and the protons of the vinyl ester group appeared at \(\delta = 4.89\) ppm (m’), \(\delta = 4.65\) ppm (m) and \(\delta = 7.21\) ppm (l). The methylene protons of the adipate moiety are observed at \(\delta = 2.28-2.47\) ppm (k,i) and \(\delta = 1.61-1.52\) ppm (j). The relative integrals of the protons of newly formed vinyl adipate chain end [(i),(j),(m) and (m’)] and the terminal methylene protons next to the benzyl group in PEG moiety [\(\delta = 4.12\) ppm (c’)] demonstrate successful functionalization with the integration ration of (i):(j):(m):(m’):(c’) as 2:4:1:1:2.
Figure 4.30. \(^1\)H NMR spectra of BzPEG\(_{1000}\) (bottom, \(M_n=1000\) g/mol, \(M_w/M_n=1.01\)) and its transesterification product (top) in DMSO-d\(_6\).

The \(^{13}\)C NMR spectrum in Figure 4.31 showed that the carbon connected to the hydroxyl group at \(\delta=60.17\) ppm (A) in the BzPEG\(_{1000}\) shifted downfield to \(\delta=63.03\) ppm (A) and the carbon signals corresponding to the vinyl group appeared at \(\delta=97.96\) ppm (L) and at \(\delta=141.17\) ppm (K), respectively. The carbon resonance of the benzyl carbons at \(\delta=71.97\) ppm (D) did not shift.
Figure 4.31. $^{13}$C NMR spectrum of the transesterification product of DVA with BzPEG$_{1000}$ (solvent: DMSO-d$_6$).

Figure 4.32 shows MALDI-ToF spectrum of the product obtained by the transesterification of DVA with BzPEG$_{1000}$ ($M_n=1000$ g/mol). There is only a single distribution with 44 $m/z$ units, corresponding to sodiated BzPEG vinyl adipate. The peak at $m/z$ 1385.928 corresponds to the sodium complex of the 25-mer of BzPEG vinyl adipate. The calculated monoisotopic mass for this peak is 1385.86 Da [$25 \times 44.03$ (C$_2$H$_4$O repeat unit) + 262.12 (C$_{15}$H$_{18}$O$_4$ end groups) + 22.99 (Na$^+$)]. There were no peaks at higher molecular weights (> 2000 Da), indicating no coupled product.
Figure 4.32. MALDI-ToF mass spectrum of the transesterification product of DVA with BzPEG$_{1000}$ ($M_n=1000$ g/mol; $M_w/M_n=1.01$).

The same reaction strategy was applied to the transesterification of DVA (3.09 g, 15.56 mmol, 2.94 mL, 40 eq. per OH in the BzPEG$_{2570}$) with higher molecular weight of BzPEG$_{2570}$ (1.0 g, 0.39 mmol, $M_n=2570$ g/mol; $M_w/M_n=1.07$) in the presence of CALB (65 mg resin @ 20wt% enzyme, $3.9 \times 10^{-4}$ mmol, $1.3 \times 10^{-4}$ mol/L) under solventless conditions.
Figure 4.33. MALDI-ToF mass spectrum of the transesterification product of DVA with BzPEG$_{2570}$ ($M_n=2570$ g/mol; $M_w/M_n=1.07$).
Figure 4.33 shows MALDI-ToF spectrum of the product resulting from the transesterification of DVA with BzPEG$_{2570}$. There are a major and a minor distribution with peaks of 44 $m/z$ units, corresponding to Na$^+$ and K$^+$ BzPEG vinyl adipate, respectively. In the expanded spectrum, the peak at $m/z = 2650.4$ corresponds to the sodium complex of the 56-mer of BzPEG vinyl adipate. The calculated monoisotopic mass for this peak is 2750.79 Da [56 $\times$ 44.03 (C$_2$H$_4$O repeat unit) + 262.12 (C$_{15}$H$_{18}$O$_4$ end groups) + 22.99 (Na$^+$)]. The minor peaks in the same region, which differ from the main series by 16 $m/z$ units, are attributed to the K$^+$ cationized BzPEG vinyl adipate (the mass difference between Na$^+$ and K$^+$ is 16 Da). There were no peaks at higher molecular weights (> 4500 Da), indicating no coupled product.

4.1.2.2.3. Transesterification of BzPEG Vinyl Adipate with 12-Bromo-1-dodecanol

BzPEG$_{1000}$ vinyl adipate (0.61 g, 0.51 mmol) was reacted with 12-bromo-1-dodecanol (0.14 g, 0.51 mmol, 1.0 eq.) in the presence of CALB (86 mg resin @ 20 wt% enzyme, 5.2 $\times$ 10$^{-4}$ mmol) in 17 mL of dried THF. The reaction was stirred for 2 hours at 50 °C (Figure 4.41).

![Figure 4.34](image_url). The transesterification of BzPEG$_{1000}$ vinyl adipate with 12-bromo-1-dodecanol in THF in the presence of CALB. [BzPEG$_{1000}$ vinyl adipate] = 0.03 mol/L, [12-bromo-1-dodecanol] = 0.03 mol/L; [CALB] = 3.0 $\times$ 10$^{-5}$ mol/L.
Figure 4.35 shows NMR spectra of the product of the transesterification of BzPEG\textsubscript{1000} vinyl adipate with 12-bromo-1-dodecanol. In the \textsuperscript{1}H NMR spectrum, the proton signals of the vinyl ester group at $\delta = 4.89$ ppm (m'), $\delta = 4.65$ ppm (m) and $\delta = 7.21$ ppm (l) disappeared and the methylene protons next to the hydroxy group from 12-bromo-1-dodecanol are observed at $\delta = 4.00$ ppm (o). The proton signals of the methylene group next to the bromine overlapped with that of the ethylene glycol repeat units at $\delta = 3.47$ ppm (q). The integration ratios of (e):(b'):(o):(i) as 1:1:1:2 demonstrate successful functionalization. The \textsuperscript{13}C NMR spectrum shows that the carbons of the vinyl group at $\delta=97.96$ ppm and at $\delta=141.17$ ppm disappeared and the carbon corresponding to the carbonyl next to the vinyl group at $\delta=170.26$ ppm shifted downfield to at $\delta=172.57$ ppm (G) after the transesterification. The carbon signal connected to bromine at $\delta=35.10$ ppm (O) appeared at the expected position. The carbon resonance at $\delta=71.97$ ppm (D) connected to the benzyl group did not shift.
Figure 4.35. NMR spectra of the transesterification product of BzPEG\textsubscript{1000} vinyl adipate with 12-bromo-1-dodecanol; (top) \textsuperscript{1}H NMR and (bottom) \textsuperscript{13}C NMR (solvent: DMSO-d\textsubscript{6}). The carbon signal (Q) in the red rectangle indicates the presence of carbon connected to a bromine group.

Figure 4.36 shows the MALDI-ToF spectrum of the transesterification product of BzPEG\textsubscript{1000} vinyl adipate with 12-bromo-1-dodecanol. There is only a single distribution of peaks, separated by 44 \textit{m/z} units, corresponding to sodiated BzPEG\textsubscript{1000}-Br. In the expanded spectrum, the peak at \textit{m/z} 1605.997 corresponds to the sodium complex of the 25-mer of BzPEG vinyl adipate. The calculated monoisotopic mass for this peak is 1605.94 Da \([25 \times 44.03 (C_2H_4O \text{ repeat unit}) + 482.20 (\text{end groups}) + 22.99 (\text{Na}^+)\)]. There were no peaks at higher molecular weights.
Figure 4.36. MALDI-ToF mass spectrum of BzPEG\textsubscript{1000} -Br.

Cationizing agent: \textit{CF}_3\textit{COONa}

Monoisotopic mass for 25-mer (Na\textsuperscript{+})

\[
m/z = 25 \times 44.03 = 482.20
\]

\[22.99\]

1605.94 Da
4.1.2.2.4. Conjugation of FA with BzPEG-Br

FA (0.08 g, 0.18 mmol) in 3 mL of anhydrous DMSO was reacted with 0.09 mL of n-BuLi (0.19 mmol, 2 M in cyclohexane). The solution was stirred for 30 minutes at room temperature. 0.2 g of BzPEG$_{1000}$-dodecyl-Br (0.13 mmol) in 1 mL of anhydrous DMSO was added in the solution using a dropping funnel under nitrogen. The solution was stirred for 24 hours at room temperature under nitrogen and dark environment. The product was precipitated in cold diethyl ether and then washed with diethyl ether/hexane mixture. The precipitate was dried in a vacuum oven.

![Conjugation of FA with BzPEG-Br using n-BuLi in anhydrous DMSO.](image)

Figure 4.37. Conjugation of FA with BzPEG-Br using n-BuLi in anhydrous DMSO. [FA] = 0.06 mol/L, [BzPEG-Br] = 0.04 mol/L; [n-BuLi] = 0.10 mol/L.

Figure 4.38 displays the NMR spectra of the conjugation product. The $^1$H NMR spectrum shows the proton resonances of the crude product. The proton signals of PEG repeat units at $\delta=3.50$ ppm (d) and FA at $\delta=7.65$ ppm (v), $\delta=6.64$ ppm (u) and $\delta=8.62$ ppm (y) appeared at the expected positions. The proton resonances of the benzyl group at $\delta=7.32$ ppm (f,g,h) and $\delta=4.48$ ppm (e) did not change after the reaction. The $^{13}$C NMR spectrum showed that the carbon connected to the bromine group at $\delta=35.10$ ppm (O) in
Figure 4.42 shifted downfield to δ=63.67 ppm (O), confirming the conjugation. Most of the carbon signals match the expected structure of BzPEG-FA and the carbon signal associated with the methylene in the benzyl group at δ = 71.98 ppm (D) did not change after the reaction. The carbons attributed to the two carboxyl groups in the glutamic moiety of the conjugated FA appeared at δ = 174.29 ppm (P) and δ = 172.65 ppm (T). Both spectra show the presence of free FA.

Figure 4.38. NMR spectra of the conjugation product of BzPEG_{1000}-Br with FA; (top) \(^1\)H NMR and (bottom) \(^{13}\)C NMR (solvent: DMSO-\(d_6\)).
In summary, the multifunctional core was successfully prepared by the CALB-catalyzed transesterification of VA with TEG and then Michael addition of diethanolamine to TEG diacrylate with/without the use of solvent. The functional PEG arms with fluorescein or FA were prepared by chemo-enzymatic reactions. For the vinyl ester functionalized PEG-Fluorescein arm, FITC was reacted with exclusively amine group of H$_2$N-PEG-OH in the presence of triethylamine via nucleophilic addition. DVA was transesterified with FITC-PEG-OH in the presence of CALB. For the FA conjugated PEG arm, DVA was first reacted with BzPEG-OH ($M_n$=1000 g/mol) in bulk in the presence of CALB. The BzPEG vinyl ester was then transesterified with 12-bromo-1-dodecanol in the presence of CALB within 2 hours. Finally, FA was conjugated with BzPEG-Br using n-BuLi as our new conjugation method. We still need to complete the deprotection of the benzyl group followed by the transesterification of DVA with FA-PEG-OH. Then the functionalized arms need to be connected to the core.

4.2. Fundamental studies

In order to get a better understanding of enzymatic catalysis, a series of fundamental studies was carried out. This section will summarize the results.

4.2.1. Transesterification of Vinyl Methacrylate with 2-(Hydroxyethyl) Acrylate

The effect of reagent and enzyme concentrations was studied in the transesterification of vinyl methacrylate (VMA) with 2-hydroxyethyl acrylate (2HEA). This reaction yielded a compound with methacrylate and acrylate terminal groups.
4.2.1.1. The effect of 2HEA Concentration

The effect of 2HEA concentration is shown in Figure 4.39. The ratio of VMA:2HEA was kept constant at 3:1. In the absence of solvent (VMA: 0.58 g, 5.17 mmol, 0.62 mL; 2HEA (0.20 g, 1.72 mmol, 0.20 mL; CALB 28.7 mg resin@20wt%, 1.7 × 10^{-4} mmol), at 2.1 mol/L 2HEA concentration the reaction was complete in 4 hours. At 0.10 mol/L 2HEA in THF, only 19% conversion was observed after 4 hours.

Figure 4.39. The effect of 2HEA concentration on the transesterification of VMA. Bulk: [VMA] = 6.31 mol/L, [HEA] = 2.10 mol/L; [CALB] = 2.0 × 10^{-4} mol/L. In THF: [2HEA] = 1.02 mol/L (THF=0.86 mL), 0.68 mol/L (THF=1.72 mL), 0.10 mol/L (THF=17.2 mL).
4.2.1.2. The Effect of Enzyme Concentration

The effect of enzyme concentration at 0.68 mol/L 2HEA concentration in THF is shown in Figure 4.40. VMA (1.45 g, 12.93 mmol, 1.55 mL) was reacted with 2HEA (0.50 g, 4.31 mmol, 0.49 mL) in dried THF (4.3 mL) at CALB concentrations ranging from $10^{-4}$ to $10^{-7}$ mol/L. At $6.6 \times 10^{-7}$ mol/L only 5.9% of conversion was observed after 4 hours. However, at $6.6 \times 10^{-4}$ mol/L enzyme concentration the reaction was complete in 4 hours.

![Figure 4.40. The effect of enzyme concentration for the transesterification of VMA. [VMA] = 2.04 mol/L; [2HEA] = 0.68 mol/L; THF = 4.3 mL in the presence of CALB. [CALB] = $6.6 \times 10^{-7}$ mol/L [0.7 mg (4.2 $\times 10^{-6}$ mmol)]; $6.6 \times 10^{-6}$ mol/L [7.0 mg (4.2 $\times 10^{-5}$ mmol)]; $6.6 \times 10^{-5}$ mol/L [70 mg (4.2 $\times 10^{-4}$ mmol)] and $6.6 \times 10^{-4}$ mol/L [700 mg (4.2 $\times 10^{-3}$ mmol)].]
4.2.1.3. The Effect of Enzyme Concentration in Bulk

The effect of enzyme concentration under solventless conditions was also investigated. Figure 4.41 shows the results. Above $2.0 \times 10^{-4}$ mol/L enzyme concentration no further improvement was found.

![Figure 4.41](image_url)

Figure 4.41. The effect of enzyme concentration on the transesterification of VMA with 2HEA in the presence of CALB. $[\text{VMA}] = 6.31$ mol/L, $[\text{2HEA}] = 2.10$ mol/L; $[\text{CALB}] = 2.0 \times 10^{-6}$ mol/L [0.7 mg ($4.2 \times 10^{-6}$ mmol)], $2.0 \times 10^{-5}$ mol/L [7.0 mg ($4.2 \times 10^{-5}$ mmol)], $2.0 \times 10^{-4}$ mol/L [70 mg ($4.2 \times 10^{-4}$ mmol)] and $6.0 \times 10^{-4}$ mol/L [210 mg ($1.3 \times 10^{-4}$ mmol)].

Figure 4.42 compares the reaction in bulk and at $[\text{2HEA}] = 1.15$ mol/L. The molar ratio of 2HEA (0.20 g, 1.72 mmol, 0.20 mL) to VMA (0.58 g, 5.17 mmol, 0.62 mL) was kept at 1:3 in the presence of CALB (28.7 mg, $1.7 \times 10^{-4}$ mmol). The progress of the reactions was monitored with TLC using ethyl acetate as an eluent and phosphomolybdic
acid as a staining agent. As shown in Figure 4.42, in THF (0.86 mL) the spot with \( R_f = 0.52 \) corresponding to 2HEA disappeared after 8 hours and a new spot attributed to the product was observed at \( R_f = 0.74 \). However, in bulk the reaction was complete in 4 hours.

![Reaction scheme and TLC monitoring of the transesterification of VMA with 2HEA in the presence of CALB.](image)

Figure 4.42. Reaction scheme and TLC monitoring of the transesterification of VMA with 2HEA in the presence of CALB. In THF: [VMA] = 3.45 mol/L; [2HEA] = 1.15 mol/L; [CALB] = 1.0 × 10^{-4} mol/L. In bulk: [VMA] = 6.30 mol/L; [2HEA] = 2.10 mol/L; [CALB] = 1.8 × 10^{-4} mol/L.

The \( ^1H \) and \( ^{13}C \) NMR spectra of the final product in DMSO-\( d_6 \), which was obtained after the filtration of enzyme and subsequent removal of VMA by a rotary evaporator at 40 °C, are given in Figure 4.43. In the \( ^1H \) NMR spectrum, the hydroxyl protons of 2HEA at \( \delta = 4.76 \) ppm disappeared and the peak corresponding to the methylene protons adjacent to the hydroxyl group shifted downfield to \( \delta = 4.35 \) ppm (e) after the reaction. The new peaks corresponding to the methyl [\( \delta = 1.85 \) ppm (f)] and vinyl
[δ=6.05 ppm (h) and δ=5.67 ppm (g)] protons of the acrylate group were observed at the expected positions.

Figure 4.43. NMR spectra of the transesterification product of VMA with 2HEA: (top) $^1$H NMR spectrum and (bottom) $^{13}$C NMR spectrum (solvent: DMSO-d$_6$).

The $^{13}$C NMR spectrum of the product also confirmed the structure of 2-(acryloyloxy)ethyl methacrylate. The carbons connected to the hydroxyl group in the
starting material at $\delta=59.47$ ppm shifted downfield to $\delta=62.17$ ppm (E) after the reaction and the carbon resonances of the methacrylate group appeared at $\delta=166.25$ ppm (F), $\delta=17.73$ ppm (I), $\delta=135.66$ ppm (G) and $\delta=125.74$ ppm (H) corresponding to carbonyl carbon, methyl carbon, $\alpha$-carbon and the vinyl carbon connected to the $\alpha$-carbon, respectively.

4.2.2. Chemoselectivity in Enzyme-catalyzed Transesterification

4.2.2.1. Transesterification of Divinyl Adipate with 11-Mercapto-1-undecanol

$\omega$-Thiol-functionalized molecules have been used in protein modifications,\textsuperscript{167} gold nanoparticle functionalization\textsuperscript{168} and cancer radiotherapy\textsuperscript{169} in biomedical applications. The thiol group is very reactive so that chemical protection is often required in the modification of free-SH bearing compounds.\textsuperscript{170} In this section, mercapto-alcohols are used to study the chemoselectivity in enzyme-catalyzed transesterification. First, the acrylation of mercapto-alcohols was attempted. The transesterification of VA with mercapto-alcohols (6-mercapto-1-hexanol, 9-mercapto-1-nonanol and 11-mercapto-1-undecanol) was performed using CALB in THF, hexane or in bulk (Figure 4.44). However, TLC showed no reaction up to 24 hours.

![Figure 4.44. Transesterification of VA (1.5 eq. per OH) with mercapto-alcohols in the presence of CALB.](image-url)
In contrast, when DVA (0.50 g, 2.52 mmol, 0.48 mL) was used as the acyl donor for the transesterification with 11-mercapto-1-undecanol (0.49 g, 2.40 mmol) in dried THF (1.2 mL) in the presence of CALB (50 mg resin @ 20wt% enzyme, $3.0 \times 10^{-3}$ mmol), the reaction was complete within 90 minutes (Figure 4.45). The progress of the reaction was monitored with TLC using hexane/THF (2/1; vol/vol) as an eluent. After 30 minutes, a new spot at $R_f = 0.66$ was detected in addition to the starting material spot at $R_f = 0.60$. After 90 minutes there was only one spot at $R_f = 0.66$.

Figure 4.45. Reaction scheme and TLC monitoring of the CALB-catalyzed Transesterification of DVA with 11-mercapto-1-undecanol in dried THF. $[\text{DVA}] = 1.50 \text{ mol/L}$, $[\text{11-mercapto-1-undecanol}] = 1.43 \text{ mol/L}; [\text{CALB}] = 1.8 \times 10^{-4} \text{ mol/L}$.

Figure 4.46 shows $^1 \text{H} \text{NMR}$ spectra of the transesterification product together with 11-mercapto-1-undecanol. The triplet methylene protons next to the hydroxyl group (h') of 11-mercapto-1-undecanol shifted to $\delta = 4.0$ ppm (h') without any change of the methylene protons (r) adjacent to the SH group. New resonances attributed to the

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vinylidene ($\delta=4.56$ ppm (b), $\delta=4.80$ ppm (a) and methine [$\delta=7.26$ ppm (c)] protons appeared with the integration ratios of (a):(b):(c):(h) as 1:1:1:2.

Figure 4.46. $^1$H NMR spectra of the transesterification product (top) of 11-mercapto-1-undecanol (bottom) with DVA (solvent: DMSO-$d_6$).

The ESI mass spectrum confirmed the formation of two transesterification products with $m/z$ 381.2 (91%) and 541.3 (9%) corresponding to 11-mercaptoundodecyl vinyl adipate and bis(11-mercaptoundodecyl) adipate, respectively. The corresponding calculated monoisotopic masses of these peaks are 381.22 Da [358.22 ($C_{19}H_{34}O_4S$, 11-
mercaptoundodecyl vinyl adipate) + 22.99 (Na\(^+\)) and 541.34 Da \([358.22 (C_{28}H_{54}O_4S_2,\text{bis(11-mercaptoundodecyl) adipate}) + 22.99 (Na^+)]\) (Figure 4.47).

![Figure 4.47. ESI mass spectrum of the transesterification products of DVA with 11-mercapto-1-undecanol (cationizing agent: NaTFA).](image)

This demonstrated the chemoselectivity of CALB, but it is not clear why DVA reacted and VMA did not.

### 4.2.3. Stereospecificity in Enzyme-catalyzed Michael Addition

#### 4.2.3.1. Michael Addition of Diethylamine to 2-(Acryloyloxy)ethyl Methacrylate

2-(Acryloyloxy)ethyl methacrylate (0.20 g, 1.15 mmol, 0.19 mL) as a \(\alpha\)-acylated, \(\omega\)-methacrylated ethylene glycol (EG) prepared in section 4.2.1 was reacted with diethylamine (0.36 g, 3.45 mmol, 0.51 mL, 3eq. relative to 2HEA) by the Michael
addition in the presence of CALB (18 mg resin @ 20 wt% enzyme, $1.1 \times 10^{-4}$ mmol) under solventless conditions (Figure 4.48). Only the acrylate group reacted, leaving the methacrylate group intact.

Figure 4.48. Enzymatic Michael addition of diethylamine to 2-(acyloyloxy)ethyl methacrylate in the presence of CALB. [diethylamine] = 4.93 mol/L, [2-(acyloyloxy)ethyl methacrylate] = 1.64 mol/L; [CALB] = $1.6 \times 10^{-4}$ mol/L.

Figure 4.49 shows the NMR spectra of the product at 30 min reaction time. The vinylidene [$\delta=6.18$ ppm] and vinyl [$\delta=6.32$ ppm and $\delta=5.97$ ppm] protons of the acrylate group disappeared while the proton resonances of the methacrylate group at $\delta = 2.73$ ppm (i) and $\delta = 0.9$ ppm (l) remained intact. In the $^{13}$C NMR spectrum, the carbons resonances corresponding to the acrylate group shifted upfield to $\delta=47.81$ ppm ($A'$) and $\delta=32.03$ ppm ($B'$) after the reaction and the carbon resonances of the methacrylate group at $\delta = 166.17$ ppm (F), $\delta = 135.64$ ppm (G), $\delta = 125.57$ ppm (H) and $\delta = 17.68$ ppm (I) did not change.
4.2.3.2. Michael Addition of Diethylamine to 2-(Acryloyloxy)ethyl Crotonate

Based on the optimization of the transesterification under solventless conditions, α-acrylated, ω-crotonated EG was prepared by the transesterification of vinyl crotonate with 2HEA (0.79 g yield, 100% conversion). Vinyl crotonate (1.45 g, 12.92 mmol, 1.54
mL, 3.0 eq. relative to 2HEA) was transesterified with 2HEA (0.50 g, 4.31 mmol, 0.49 mL) in the presence of CALB (72 mg resin 20 wt% enzyme, $4.3 \times 10^{-4}$ mmol) under solventless conditions (Figure 4.50).

![Figure 4.50. Transesterification of vinyl crotonate with 2HEA in the presence of CALB under solventless conditions. [vinyl crotonate] = 6.36 mol/L, [2HEA] = 2.12 mol/L; [CALB] = $2.1 \times 10^{-4}$ mol/L.](image)

The structure of the transesterification product was confirmed by $^1$H and $^{13}$C NMR spectroscopy (Figure 4.51). The hydroxyl protons of 2HEA at $\delta=4.76$ ppm disappeared and the peak corresponding to the methylene protons adjacent to the hydroxyl group shifted downfield to $\delta=4.35$ ppm (e). The new peaks corresponding to the methyl [$\delta=1.81$ ppm (h)] and vinyl [$\delta=6.93$ ppm (g) and $\delta=5.85$ ppm (f)] protons of the crotonate group were observed at the expected positions. The $^{13}$C NMR spectrum of the transesterification product also confirmed the structure of 2-(acryloyloxy)ethyl crotonate. The carbons connected to the hydroxyl group in the starting material at $\delta=59.47$ ppm shifted downfield to $\delta=62.20$ ppm (E) after the reaction and the carbon resonances of the crotonate group [$\delta=165.27$ ppm (F), $\delta=17.29$ ppm (I), $\delta=145.07$ ppm (G) and $\delta=122.03$ ppm (H)] and the acrylate group [$\delta=165.27$ ppm (C), $\delta=130.09$ ppm (A) and $\delta=128.02$ ppm (B)] appeared at the expected position, confirming the successful functionalization.
To study the stereoselectivity diethylamine (0.36 g, 3.45 mmol, 0.38 mL, 3eq.) was reacted with 2-(acyloxyloxy)ethyl crotonate (0.2 g, 1.15 mmol, 0.19 mL) by the CALB (18 mg resin @ 20wt% enzyme, $1.1 \times 10^{-4}$ mmol)-catalyzed Michael addition (Figure 4.52).
Figure 4.52. Enzymatic Michael addition of diethylamine to 2-(acryloyloxy)ethyl crotonate in the presence of CALB under solventless conditions. [diethylamine] = 6.05 mol/L, [2-(acryloyloxy)ethyl crotonate] = 2.02 mol/L; [CALB] = 1.9 \times 10^{-4} \text{ mol/L}.

Figure 4.53. NMR spectra of Michael addition product of diethylamine to 2-(acryloyloxy)ethyl crotonate: (top) $^1$H NMR spectrum and (bottom) $^{13}$C NMR spectrum (solvent: DMSO-$d_6$).
The $^1$H NMR spectrum confirmed that the Michael addition to the acrylate group was complete within 30 min, while the crotonate group remained intact (Figure 4.53).

4.2.3.3. Michael Addition of Diethanolamine to TEG dimethacrylate

VA is more expensive than VMA so we explored the use of this latter. TEG-dimethacrylate was prepared as described in Section 4.3.1 (1.69 g yield, 100% conversion). The Michael addition of DEA (1.19 g, 12.14 mmol, 1.08 mL) to TEG dimethacrylate (1.83 g, 5.52 mmol, 1.69 mL) in anhydrous DMSO (2.76 mL) in the presence of CALB (184 mg resin @ 20 wt% enzyme, $1.1 \times 10^{-3}$ mmol) was monitored by TLC [eluent: Hexane/THF (1/1; vol/vol, staining agent: phosphomolybdic acid] for 48 hours (Figure 4.54). The spot at $R_f=0.78$ corresponding to TEG dimethacrylate could still be observed after 48 hours.

Figure 4.54. Reaction scheme and TLC monitoring of the CALB-catalyzed Michael addition of diethanolamine to TEG dimethacrylate in DMSO. [DEA] = 2.20 mol/L, [TEG dimethacrylate] = 1.00 mol/L; [CALB] = $2.0 \times 10^{-4}$ mol/L.
NMR spectroscopy also revealed incomplete conversion. Figure 4.55 shows the $^1$H NMR spectrum of the product at 24 hours. The signals of the methyl protons next to the carbonyl group at $\delta = 1.00$ ppm (c’) and the methylene protons next to the ester linkage at $\delta = 4.11$ ppm (d’) are observed from the Michael addition product. However, the proton of the unreacted methacrylate group in the TEG dimethacrylate are seen at $\delta = 6.04$ ppm (a), $\delta = 5.76$ ppm (b) and $\delta = 1.74$ ppm (c). Based on the integration ratio of (c) : (c’), only 12.1% conversion was achieved. No further progress could be detected by NMR after 48 hours. However, when TEG diacrylate was used in the Michael addition of diethanolamine (section 4.1.1.3), the reaction was quantitative within 2 hours. This indicates stereospecificity. It is in good agreement with previous studies, performed by Dr. Sen from our research group. He prepared vinyl ester functionalized thymine using the CALB-catalyzed transesterification of VMA and VA in DMSO. A lower conversion (63%) was observed with VMA compared to quantitative Michael addition with VA, likely due to the steric hindrance created by the methyl group.\cite{149}

Figure 4.55. $^1$H NMR spectrum of the Michael addition product of diethanolamine to TEG dimethacrylate at $t = 24$ hours (solvent: DMSO-d$_6$).
4.2.4. Vinyl-ester functionalization of TEGs

4.2.4.1. Transesterification of Divinyl Adipate with TEG in THF

The transesterification of DVA with TEG in THF in the presence of CALB was investigated (Figure 4.56). DVA (3.06 g, 14.44 mmol, 2.91 mL, 3.0 eq. per OH in TEG) was reacted with TEG (1.00 g, 5.15 mmol, 0.89 mL) in dried THF (2.6 mL) in the presence of CALB (86 mg resin @ 20 wt% enzyme, 5.2 × 10⁻⁴ mmol). The reaction was stirred for 40 min at 50 °C. The progress of reaction was monitored by TLC [eluent: hexane/THF (1/1; vol/vol), phosphomolybdic acid] and ESI MS. The spot of HO-TEG-OH disappeared within 30 min. The reaction was stopped at 40 min. Silica gel column chromatography was used to remove the excess of DVA using hexane/THF (5/1; vol/vol).

![Reaction scheme and TLC monitoring of the transesterification of DVA with TEG in THF in the presence of CALB.](image)

Figure 4.56. Reaction scheme and TLC monitoring of the transesterification of DVA with TEG in THF in the presence of CALB. [DVA] = 2.26 mol/L, [TEG] = 0.80 mol/L; [CALB] = 8.1 × 10⁻⁵ mol/L.

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In Figure 4.57, the ESI mass spectra show the transesterification products after (a) 30 minutes and (b) 40 minutes, respectively. At 30 minutes, there was about 2% of mono-functionalized TEG-OH and 98% difunctional product. The ESI mass spectrum at 40 minutes reaction time confirmed the formation of a single product with \( m/z \) 525.3 \([\text{M+Na}^+]\), which is in good agreement with the calculated monoisotopic mass of this ion, 325.25 Da \([4 \times 44.03 \text{ (C}_2\text{H}_4\text{O repeat unit)} + 326.14 \text{ (C}_1\text{H}_2\text{O}_7 \text{ end groups)} + 22.99 \text{ (Na}^+\text{)}\)].

Figure 4.57. ESI mass spectra of the transesterification product at (a) 30 minutes and (b) 40 minutes reaction time.

Figure 4.58 shows \(^1\text{H}\) and \(^{13}\text{C}\) NMR spectra of vinyl adipate-TEG-vinyl adipate as the transesterification product in DMSO-d$_6$. In the \(^1\text{H}\) NMR spectrum, the proton resonances at \(\delta=4.55\) ppm corresponding to the -OH protons of HO-TEG-OH disappeared and the peak of the methylene protons adjacent to hydroxyl group shifted downfield from \(\delta=3.50\) to \(\delta=4.22\) ppm (b) after the reaction. New resonances attributed to
the vinylidene (δ=4.87 (e), δ= 4.65 (e’)), and methine (δ=7.24 (f)) appeared with the integration ratios of (b):(f):(e):(e’) as 2:1:1:1. The $^{13}$C NMR spectrum showed that the carbons connected to the hydroxyl group at δ=60.13 ppm in the HO-TEG-OH shifted downfield to δ=63.09 ppm (B) after 30 min reaction and new carbon resonances of the vinyl groups [δ=141.17 ppm (F) and δ=97.76 ppm (E)] and carbonyl carbons resonances of adipic ester groups [δ=172.60 ppm (G) and δ=170.15 ppm (K)] appeared at the expected positions, confirming the structure of the product.

Figure 4.58. NMR spectra of the transesterification product of DVA with TEG: (top) $^1$H NMR spectrum and (bottom) $^{13}$C NMR spectrum (solvent: DMSO-d$_6$).
4.2.4.2. The Effect of Divinyl Adipate Concentration on the Transesterification with TEG under Solventless Conditions

To investigate the effect of DVA concentration on the transesterification with TEG, different molar equivalents of DVA [1.5 eq. (0.61 g, 3.09 mmol, 0.58 mL), 3.0 eq. (1.22 g, 6.18 mmol, 1.16 mL), 5.0 eq. (2.04 g, 10.29 mmol, 1.94 mL) and 10.0 eq. (4.08 g, 20.59 mmol, 3.98 mL) eq. per OH in TEG] were reacted with each 0.2 g of TEG (1.03 mmol, 0.18 mL) in the presence of CALB (17 mg resin @ 20 wt% enzyme, $1.0 \times 10^{-4}$ mmol) for 1 hour at 50 °C (Figure 4.59). The excess of DVA was removed by silica gel chromatography using the mixture of hexane/THF (5/1; vol/vol) as the eluent.

![Figure 4.59. Transesterification of DVA with TEG in the presence of CALB under solventless conditions. [DVA] = 3.60 mol/L 1.5 eq., 4.61 mol/L 3.0 eq., 4.85 mol/L 5.0 eq., 4.95 mol/L 10.0 eq.; [TEG] = 1.36 mol/L 1.5 eq., 0.77 mol/L 3.0 eq., 0.49 mol/L 5.0 eq., 0.25 mol/L 10.0 eq.; [CALB] = $1.3 \times 10^{-4}$ mol/L 1.5 eq., $7.6 \times 10^{-5}$ mol/L 3.0 eq., $4.8 \times 10^{-5}$ mol/L 5.0 eq., $2.5 \times 10^{-5}$ mol/L 10.0 eq.](image)

The products were analyzed by MALDI-ToF MS using dithranol matrix and NaTFA as cationizing salt in a 10:1:2 ratio of matrix : cationizing salt : sample. When 3.0 eq. of DVA per OH in TEG was used in the reaction, 54.3% oligomerized products were found (Figure 4.60). Within each distribution the peaks were separated by the C$_{14}$H$_{24}$O$_7$ repeat unit (304.2 Da), corresponding to TEG-adipate.
Figure 4.60. MALDI-ToF mass spectrum of the transesterification product of DVA (6.18 mmol, 3.0 eq. per -OH in TEG) with TEG (1.03 mmol) at 1 hour (cationizing salt: NaTFA).

Table 4.1. The effect of DVA concentration on oligomerization of TEG.

<table>
<thead>
<tr>
<th>Composition (%)</th>
<th>Molar ratio of DVA per OH</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td>Divinyl-TEG</td>
<td>42.5</td>
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<tr>
<td>Dimer</td>
<td>35.5</td>
</tr>
<tr>
<td>Trimer</td>
<td>17.7</td>
</tr>
<tr>
<td>Tetramer</td>
<td>3.8</td>
</tr>
<tr>
<td>Pentamer</td>
<td>0.5</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
</tr>
</tbody>
</table>
However, the amount of oligomerized products decreased with increasing DVA concentration (Table 4.1). When 1.5 eq. of DVA per OH in TEG was used in the reaction, 42.5% of TEG divinyl diadipate was observed with 56.5% of oligomerized products. At 10 eq. of DVA, only 18.4% of oligomerized products were obtained.

4.2.5. Vinyl-ester Functionalization of PEGs

4.2.5.1. The Effect of Divinyl Adipate Concentration on the Transesterification with PEG under Solventless Conditions

The transesterification of DVA with PEGs ($M_n = 1000$ g/mol and 2000 g/mol) was also investigated. As discussed in section 4.2.5.2, when DVA (10.0 eq. per OH in TEG) was reacted with TEG in bulk, 18.4% of oligomerized products were observed in the MALDI-ToF spectrum. The same synthetic procedure was applied to the transesterification of DVA (3.96 g, 20.0 mmol, 3.77 mL, 10.0 eq. per OH) with PEG (1.00 g, 1.0 mmol, $M_n = 1000$ g/mol; $M_w/M_n = 1.08$) in the presence of CALB (100 mg resin @ 20 wt% enzyme, $6.0 \times 10^{-4}$ mmol) under solventless conditions (Figure 4.60).

![Figure 4.61. Transesterification of DVA with PEG ($M_n = 1000$ g/mol, $M_w/M_n = 1.08$) in the presence of CALB under solventless conditions. [DVA] = 5.31 mol/L, [PEG] = 0.27 mol/L; [CALB] = $1.6 \times 10^{-4}$ mol/L.](image-url)
Figure 4.62. MALDI-ToF mass spectra of the transesterification of DVA (10.0 eq. per OH in PEG) with PEG at $t = 2$ and 4 hours.
MALDI-ToF mass spectrometry was used to monitor the progress of the reaction (Figure 4.62). Two aliquots were taken at \( t = 2 \) and 4 hours. It was found that at \( t = 2 \) hours the transesterification reaction was not complete and there was a mixture of monosubstituted PEG (8.9%), disubstituted PEG (86.9%) and coupled PEG (4.2%). The monosubstituted PEG disappeared at \( t = 4 \) hours and only two sets of peaks were observed. The main series corresponds to the sodiated PEG divinyl diadipate (98.0 %) and the minor series in the high mass region belongs to the sodiated coupled PEG product (2.0%) at high mass region. The representative peak at \( m/z \) 1362.0 in the major distribution corresponds to the sodiated 23-mer of PEG divinyl diadipate. The calculated monoisotopic mass for this peak \([m/z = 23 \times 44.03 \text{ (C}_2\text{H}_4\text{O repeat unit)} + 326.14 \text{ (C}_{16}\text{H}_{22}\text{O}_7 \text{end groups}) + 22.99 \text{ (Na}^+)\]) is 1361.82 Da.

The same reaction was repeated with a higher molecular weight PEG (1.00 g, 0.5 mmol, \( M_n = 2000 \text{ g/mol, } M_w/M_n = 1.20 \)) using 10 eq. of DVA (1.98 g, 10.0 mmol, 1.89 mL) per OH in PEG in the presence of CALB (50 mg, \( 3.0 \times 10^{-4} \) mmol, \( 1.6 \times 10^{-4} \) mol/L). Figure 4.63 shows NMR spectra of the transesterification product. The methylene protons adjacent to the hydroxy end group shifted downfield to \( \delta = 4.12 \) ppm (b) and new resonances attributed to the vinylidene (\( \delta = 4.87 \) (e), \( \delta = 4.65 \) (e')), and methine (\( \delta = 7.26 \) (f)) protons appeared. The \(^{13}\text{C} \) NMR spectrum showed that the resonance of the carbons connected to the hydroxyl groups in the starting PEG shifted downfield to \( \delta = 63.05 \) ppm (B) and new carbon resonances of the vinyl groups (F and E) and carbonyl carbons of the adipic ester groups (G and K) appeared at the expected positions after 4 hours, confirming the structure of the product.
Figure 4.63. NMR spectra of the transesterification product of DVA with PEG \( (M_n=2000 \text{ g/mol}; M_w/M_n=1.20) \): (top) \(^{13}\)C NMR and (bottom) \(^1\)H NMR (solvent: DMSO-d\(_6\)).

In the MALDI-ToF mass spectrum (Figure 4.64), two sets of distributions corresponding to the sodiated and potassiated divinyl ester of PEG were observed. The representative peak at \( m/z \) 2198.41 in the major distribution corresponds to the sodiated 42-mer of PEG divinyl diadipate. The calculated monoisotopic mass for this peak \([m/z = 42 \times 44.03 (C_2H_4O \text{ repeat unit}) + 326.14 (C_{16}H_{22}O_7 \text{ end groups}) + 22.99 (Na^+)\] is 2198.39 Da. There were no peaks at higher molecular weights (\( > 3000 \text{ Da} \)).
Figure 4.64. MALDI- ToF mass spectrum of the transesterification product of DVA with PEG ($M_n = 2000$ g/mol; $M_n/M_w = 1.20$).
4.2.5.2. The Effect of DVA Concentration on Coupling using PEG Monomethyl Ether

Poly(ethylene glycol) monomethyl ether (MPEG-OH) was functionalized by CALB-catalyzed transesterification of DVA in bulk as described in Figure 4.65. MPEG-OH (1.00 g, 0.91 mmol, $M_n = 1100$ g/mol; $M_w/M_n = 1.09$) was reacted with different concentration of DVA [1.5 eq. (0.27 g, 1.37 mmol, 0.26 mL), 3.0 eq. (0.54 g, 2.73 mmol), 5.0 eq. (0.90 g, 4.55 mmol, 0.86 mL) and 10 eq. (1.80 g, 9.1 mmol, 1.71 mL)] in the presence of CALB (100 mg, $6.0 \times 10^{-4}$ mmol) under solventless conditions.

![Figure 4.65. Transesterification of DVA with MPEG-OH in the presence of CALB under solventless conditions.](image)

The NMR spectra of the transesterification product with DVA (5.0 eq. per OH) are shown in Figure 4.66. The resonance at $\delta=4.5$ ppm, corresponding to the -OH proton of MPEG-OH disappeared and the peak of the methylene protons adjacent to hydroxyl group appeared at $\delta=4.14$ ppm (b) after the reaction. New resonances attributed to the vinylidene ($\delta=4.64$ (l), $\delta=4.85$ (l') and $\delta=7.18$ (k)) protons appeared with the integration ratios of (b):(l):(l'):(k) as 2:1:1:1. The $^{13}$C NMR spectrum showed that the carbon connected to the hydroxyl group at $\delta=60.1$ ppm in the MPEG-OH shifted downfield to $\delta=63.4$ ppm (A) and the carbon signals corresponded to the vinyl group appeared at $\delta=98.1$ ppm (M) and at $\delta=141.3$ ppm (L), respectively.
Figure 4.66. NMR spectra of the transesterification product of DVA (5.0 eq. per OH) with MPEG-OH ($M_n=1100$ g/mol, $M_w/M_n=1.09$): (top) $^1$H NMR spectrum and (bottom) $^{13}$C NMR spectrum (solvent: DMSO-d$_6$).
Figure 4.67. MALDI-ToF mass spectrum of the transesterification product of DVA with MPEG-OH.
The MALDI-ToF spectrum in Figure 4.67 shows three distributions. In the low molecular weight region, \( m/z \) 800-2000, there is one major and one minor distribution, corresponding to \( \text{Na}^+ \) and \( \text{K}^+ \) MPEG vinyl adipate, respectively. In the expanded spectrum, the peak at \( m/z \) 1309.79 corresponds to the sodium complex of the 25-mer of MPEG vinyl adipate. The calculated monoisotopic mass for this peak is 1309.75 Da \([25 \times 44.03 \text{ (C}_2\text{H}_4\text{O} \text{ repeat unit)} + 186.01 \text{ (C}_8\text{H}_{10}\text{O}_3 \text{ end groups}) + 22.99 \text{ (Na}^+)\])]. Within the major series, the peaks are separated by 44 Da, corresponding to an ethylene glycol repeating unit. The minor peaks in the same region differ from the main series by 16 \( m/z \) units. They are attributed to the \( \text{K}^+ \) cationized PEG vinyl adipate (the mass difference between \( \text{Na}^+ \) and \( \text{K}^+ \) is 16 Da). In the high molecular weight region, \( m/z \) 2000-2800, 1.0\% of MPEG-MPEG coupled product was observed.

The effect of DVA concentration on coupling on the transesterification of DVA with MPEG-OH \((M_n=1100 \text{ g/mol})\) was investigated. The amount of dimeric products decreased with increasing DVA concentration (Figure 4.68). When 1.5 eq. of DVA per OH in MPEG was used in the reaction, 35\% of MPEG-MPEG was observed. At 10 eq. of DVA, only 0.4 \% of coupled products were observed. No coupled product was found when MPEG-OH \((M_n=2000 \text{ g/mol})\) was used for the reaction at 5 eq. of DVA. There were 1.0\% of coupled products in the reaction of MPEG-OH \((M_n=750 \text{ g/mol})\) with 5 eq. of DVA.
In conclusion, vinyl ester functionalized PEGs were successfully prepared by the CALB-catalyzed transesterification of DVA with PEGs. Increasing the DVA concentration decreased the extent of coupling. At higher PEG MWs no coupling was observed.

4.3. Enzymatic Functionalization of TEGs

Based on the fundamental studies in section 4.2, TEGs were enzymatically functionalized with vinyl esters under solventless conditions.

4.3.1. Transesterification of Vinyl Methacrylate with TEG

TEG (1.00 g, 5.15 mmol, 0.89 mL) in the liquid state was enzymatically methacrylated by the transesterification of VMA (1.76 g, 15.70 mmol, 1.89 mL, 1.5 eq.)
per OH in TEG) using CALB (85 mg resin @ 20 wt% enzyme, $5.1 \times 10^{-4}$ mmol) under solventless conditions (Figure 4.69). After 2 hours a new spot at $R_f = 0.68$ corresponding to TEG dimethacrylate was detected and very weak TEG spot at $R_f = 0.02$ was still present on the TLC using hexane/THF (1/1; vol/vol). There was only one spot at $R_f = 0.68$ after four hours reaction. The enzyme was filtered out using a syringe filter and the yellowish liquid product was dried in a vacuum oven (1.69 g yield, 100% conversion).

Figure 4.69. Reaction scheme and TLC monitoring of the transesterification of VMA with TEG in the presence of CALB under solventless conditions. [VMA] = 5.65 mol/L, [TEG] = 1.85 mol/L; [CALB] = $1.8 \times 10^{-4}$ mol/L.

Figure 4.70 displays the NMR spectra of the transesterification product. The resonance at $\delta = 4.55$ ppm, corresponding to the -OH protons of HO-TEG-OH disappeared and the peak of the methylene protons adjacent to hydroxyl group shifted downfield from $\delta = 3.50$ to $\delta = 4.24$ ppm (b) after the reaction. New resonances attributed to the vinylidene ($\delta = 5.76$ (e'), $\delta = 6.04$ (e) and methyl ($\delta = 1.74$ ppm (f)) protons appeared with the integration ratios of (b):(f):(e):(e') as 2:3:1:1. The $^{13}$C NMR spectrum showed that the
carbons connected to the hydroxyl group at $\delta=60.13$ ppm in the HO-TEG-OH shifted downfield to $\delta=63.41$ ppm (B) and the carbon signals associated with the methacrylate group at $\delta = 17.80$ ppm (G) $\delta = 135.87$ ppm (F), $\delta = 125.45$ ppm (E) and $\delta = 166.39$ ppm (H) appeared after 4 hours reaction.

Figure 4.70. NMR spectra of the transesterification product of VMA with TEG: (top) $^1$H NMR spectrum and (bottom) $^{13}$C NMR spectrum (solvent: DMSO-d$_6$).

The ESI mass spectrum confirmed the formation of a single product with $m/z$ 353.2 [M+Na$^+$], which is in good agreement with the calculated monoisotopic mass of
this ion, 353.19 Da [4×44.03 (C$_2$H$_4$O repeat unit) + 154.08 (C$_8$H$_{10}$O$_3$ end groups) + 22.99 (Na$^+$)] (Figure 4.71).

Figure 4.71. ESI mass spectrum of the transesterification product of VMA with TEG (cationizing salt: NaTFA).

4.3.2. Transesterification of Vinyl Crotonate with TEG

TEG dicrotonate (3.40 g yield, 100% conversion) was also quantitatively prepared by the transesterification of vinyl crotonate (3.46 g, 30.89 mmol, 3.68 mL, 1.5 eq. per OH in the TEG) with TEG (2.00 g, 10.29 mmol, 1.78 mL) in the presence of CALB (171 mg resin @ 20 wt% enzyme, 1.0 × 10$^{-3}$ mmol) without the use of solvents within 4 hours (Figure 4.72).

Figure 4.72. Transesterification of vinyl crotonate with TEG in the presence of CALB under solventless conditions. [vinyl crotonate] = 5.66 mol/L, [TEG] = 1.88 mol/L; [CALB] = 1.9 × 10$^{-4}$ mol/L.
Figure 4.73 shows the $^1$H and $^{13}$C NMR spectra of the transesterification product. The triplet peak of the methylene protons adjacent to hydroxyl group shifted downfield from δ=3.50 to δ=4.18 ppm (b) after the reaction. New proton resonances attributed to the methyl [δ=1.85 ppm (e)] and vinyl [δ=6.90 ppm (f) and δ=5.88 ppm (g)] of the crotonate group were observed at the expected positions. The $^{13}$C NMR spectrum showed that the
carbons connected to the hydroxyl groups in the starting material shifted downfield to δ=63.01 ppm (B) and the carbon signals associated with the crotonate group [δ=165.48 ppm (H), δ=145.26 ppm (G), δ=122.11 ppm (F) and δ=17.55 ppm (E)] appeared at the expected position, confirming the successful functionalization.

4.3.3. Transesterification of Vinyl Methacrylate with TEG Monobenzyl Ether

TEG monobenzyl ether (BzTEG-OH) was reacted with VMA and vinyl crotonate by the CALB-catalyzed transesterification under solventless conditions. The benzyl ether group can be removed by a simple deprotection procedure and monofunctionalized TEG-OH can be obtained. The functionalization procedure for the methacrylation involved the transesterification of VMA (0.59 g, 5.28 mmol, 0.63 mL, 3.0 eq. per OH in the BzTEG-OH) with BzTEG-OH (0.50 g, 1.76 mmol, 0.45 mL) in the presence of CALB (29 mg resin @ 20 wt% enzyme, 1.7 × 10⁻⁴ mmol) (Figure 4.74). A new spot at R_f = 0.47 was detected together with the BzTEG-OH spot at R_f=0.02 after 1 hour on the TLC using ethyl acetate/hexane (2/1; vol/vol). There was only one spot at R_f=0.47 after 2 hours reaction. The enzyme was filtered out using a syringe filter and the yellowish liquid product was dried in a vacuum oven (0.61 g yield, 100% conversion).
Figure 4.74. Reaction scheme and TLC monitoring of the enzymatic methacrylation of BzTEG-OH with VMA in the presence of CALB under solventless conditions and TLC monitoring. \([\text{VMA}] = 4.89 \ \text{mol/L}, \ [\text{BzTEG-OH}] = 1.63 \ \text{mol/L}; \ [\text{CALB}] = 1.6 \times 10^{-4} \ \text{mol/L}.

\(^1\text{H}\) and \(^{13}\text{C}\) NMR spectra verified the expected structure of BzTEG methacrylate. Figure 4.75 shows NMR spectra of the transesterification product of VMA with BzTEG-OH. The peaks corresponding to the vinyl \([\delta = 6.04 \ \text{ppm} \ (j) \ \text{and} \ [\delta = 5.65 \ \text{ppm} \ (j')]\) and methyl protons \([\delta = 1.88 \ \text{ppm} \ (i)]\) of the methacrylate end group appeared after 2 hours of the reaction. The methylene protons adjacent to the methacrylate group at \([\delta = 4.20 \ \text{ppm} \ (b)]\) and next to the benzyl group at \([\delta = 4.48 \ \text{ppm} \ (e)]\) are observed at the expected positions with the integral ratio of 1:1:3:2:2 \((j:j':i:b:e)\) confirming the successful incorporation of the methacrylate end group and the intact benzyl ether group at the other chain end. In the \(^{13}\text{C}\) NMR spectrum, the carbon connected to the hydroxyl group of the starting material at \([\delta = 60.35 \ \text{ppm}]\) shifted to downfield at \([\delta = 63.65 \ \text{ppm} \ (A)]\) after the reaction and the carbon resonances of the methacrylate group appeared at \([\delta = 125.62 \ \text{ppm}].\)
(L), δ=135.78 ppm (K), δ=17.87 ppm (J) and δ=166.43 ppm (I). The carbon signals of the benzyl group at δ=72.00 ppm (D), δ=138.45 ppm (E), δ=127.37 ppm (H,G) and δ=128.10 ppm (F) did not change at all, indicating that the benzyl group remained intact.

Figure 4.75. NMR spectra of the transesterification product of VMA with BzTEG-OH: (top) $^1$H NMR spectrum and (bottom) $^{13}$C NMR spectrum (solvent: DMSO-d$_6$).
4. 3.4. Transesterification of Vinyl Crotonate with TEG Monobenzyl Ether

Vinyl Crotonate (0.59 g, 5.28 mmol, 0.63 mL, 3.0 eq. per OH in the BzTEG-OH) was reacted with BzTEG-OH (0.50 g, 1.76 mmol, 0.45 mL) in the presence of CALB (29 mg resin @ 20 wt% enzyme, $1.7 \times 10^{-4}$ mmol) in bulk for 2 hours (Figure 4.76).

![Figure 4.76. Enzymatic crotonation of BzTEG-OH with vinyl in the presence of CALB under solventless conditions. [Vinyl crotonate] = 4.89 mol/L, [BzTEG-OH] = 1.63 mol/L; [CALB] = $1.6 \times 10^{-7}$ mol/L.](image)

Figure 4.76 shows $^1$H and $^{13}$C NMR spectra of the crotonation product. The proton resonances attributed to the vinyl [$\delta = 6.93$ (i) and $\delta = 5.90$ ppm (j)] and methyl protons [$\delta = 1.82$ ppm (k)] of the crotonate end group appeared at the expected positions after 2 hours reaction. The methylene protons [$\delta = 4.18$ ppm (b)] adjacent to the methacrylate group as well as the methylene protons next to the benzyl group at $\delta = 4.48$ ppm (e) are observed at the expected positions with the integral ratio of 3:2:2 (k:b:e) confirming the successful incorporation of the crotonate end group and leaving the benzyl ether intact. The $^{13}$C NMR spectrum shows that the carbon connected to the hydroxyl group in the starting material at $\delta = 60.35$ ppm shifted downfield at $\delta = 63.00$ pm (A) and the carbon resonances of the crotonate group appeared at $\delta = 17.55$ ppm (L), $\delta = 122.11$ ppm (K), $\delta = 145.22$ ppm (J) and $\delta = 165.47$ ppm (I).
Figure 4.77. NMR spectra of the transesterification product of vinyl crotonate with BzTEG: (top) $^1$H NMR spectrum and (bottom) $^{13}$C NMR spectrum (solvent: DMSO-d$_6$).
4.3.5. Synthesis of (HO)$_4$-TEG-(OH)$_4$

As described in section 4.1, we successfully made the four-functional TEG core of the desired four arm star PEG using enzyme catalysis. In order to get more arms (HO)$_4$-TEG-(OH)$_4$ was synthesized. (HO)$_2$-TEG-(OH)$_2$ was reacted with a vinyl-acrylate functional linker, followed by the Michael addition of DEA to the (Acr)$_2$-TEG-(Acr)$_2$ in the presence of CALB. This section will describe the results.

4.3.5.1. Synthesis of a Vinyl-acrylate Functionalized Linker

Our studies established that acrylate functionality was necessary for effective Michael addition for the synthesis of [(HO)$_2$-TEG-(OH)$_2$]. Unfortunately, vinyl acrylate became unavailable in the middle of our studies. Therefore we set out to synthesize our own vinyl-acrylate functionalized linker.

DVA (2.00 g, 10.09 mmol, 1.90 mL) in 5.0 mL of dried THF was reacted with 2HEA (1.17 g, 10.09 mmol, 1.16 mL) in the presence of CALB (200 mg resin @ 20 wt% enzyme, $1.2 \times 10^{-3}$). The progress of the reaction was monitored with TLC using hexane/THF (3/1; vol/vol) as the eluent mixture (Figure 4.78). The product was purified by silica gel column chromatography using hexane and THF mixture (5/1; vol/vol). The liquid product was dried using a vacuum oven (2.1 g yield, 77% conversion). The reaction was complete after 3 hours, as the spot at $R_f = 0.36$ corresponding to 2HEA disappeared and new spots appeared at $R_f = 0.57$ and 0.75. The product was analyzed by $^1$H and $^{13}$C NMR spectroscopy (Figure 4.79).
The hydroxyl protons at δ=4.76 ppm from 2HEA disappeared and the signals of the methylene protons adjacent to hydroxyl group shifted downfield to δ=4.35 ppm (r,s) after the reaction. The new peaks corresponding to the vinylidene [δ=4.87 ppm (j) and δ=4.65 ppm (k)] and methine [δ=7.26 ppm (l)] protons of the vinyl ester group appeared with the integration ratios of (r,s):(j):(k):(l) as 4:1:1:1. The $^{13}$C NMR spectrum of the transesterification product also confirmed the structure of 2-(acryloyloxy)ethyl vinyl adipate. The carbons connected to the hydroxyl group in the starting material at δ=59.47 ppm shifted downfield to δ=62.17 ppm (R,S) after the reaction and the carbon resonances of the vinyl ester group appeared at δ=97.70 ppm (K), δ=141.15 ppm (J) and δ=165.26 ppm (L'). The carbons corresponding to the acrylate group appeared at δ=127.95 ppm.
(O), \( \delta=131.68 \) ppm (P) and \( \delta=170.10 \) ppm (Q), confirming the structure of the vinyl-acrylate product.

![NMR spectra](image)

Figure 4.79. NMR spectra of the transesterification product of DVA with 2HEA: (top) \(^1\)H NMR spectrum and (bottom) \(^{13}\)C NMR spectrum (solvent: DMSO-\(d_6\)).

The ESI mass spectrum (Figure 4.80) showed the presence of about 10\% of coupled product, together with the monofunctional target with \( m/z \) 293.0 [M+Na\(^+\)],
which is in good agreement with the calculated monoisotopic mass of this ion, 293.10 Da [270.11 (C_{13}H_{18}O_6) + 22.99 (Na^+)].

![Graph](image)

Figure 4.80. ESI mass spectrum of the transesterification product of DVA with 2HEA.

4.3.5.2. Transesterification of 2-(Acryloyloxy)ethyl Vinyl Adipate with (HO)$_2$-TEG-(OH)$_2$

The next step was to prepare the acrylate-functionalized TEG from (HO)$_2$-TEG-(OH)$_2$ using 2-(acryloyloxy)ethyl vinyl adipate as the vinyl-acrylate functional linker (Figure 4.81). Tetra-acrylated TEG was prepared by the transesterification of 2-(acryloyloxy)ethyl vinyl adipate (1.62 g, 5.85 mmol, 1.5 eq. per OH) with (HO)$_2$-TEG-(OH)$_2$ (0.50 g, 0.98 mmol) in dried THF (4.8 mL) in the presence of CALB (65 mg resin @ 20 wt% enzyme, $3.9 \times 10^{-4}$ mmol). According to the TLC monitoring using hexane/THF (1/1; vol/vol) mixture with phosphomolybdic acid, the spot at $R_f = 0.00$ corresponding to (HO)$_2$-TEG-(OH)$_2$ disappeared after 18 hours. The yellowish viscous
product was obtained by drying using a rotary evaporator to remove the THF. The product was dried in a vacuum oven (1.28 g yield, 84.7 % conversion).

Figure 4.81. Transesterification of 2-(acryloyloxy)ethyl vinyl adipate with (HO)$_2$-TEG-(OH)$_2$ in THF in the presence of CALB. [2-(acryloyloxy)ethyl vinyl adipate] = 1.80 mol/L, [(HO)$_2$-TEG-(OH)$_2$] = 0.20 mol/L; [CALB] = $8.1 \times 10^{-5}$ mol/L.

Figure 4.82 shows $^1$H NMR spectrum of the transesterification product in DMSO-$d_6$. The peak of the methylene protons adjacent to the hydroxyl groups in the (HO)$_2$-TEG-(OH)$_2$ shifted to $\delta=4.00$ ppm (h) after the reaction and new resonances attributed to the vinylidene [$\delta=6.18$ ppm (p)] and vinyl [$\delta=6.32$ ppm (o) and $\delta=5.97$ ppm (o')] protons appeared at the expected position. The integration ratios of $[(r+s+b+h):(p):(q):(r) = 7:1:1:1]$. 
In the $^{13}$C NMR spectrum, the carbon resonance next to the hydroxyl groups at $\delta = 59.25$ ppm shifted downfield to $\delta = 61.76$ ppm (I) and the carbon signals associated with the acrylate group [$\delta = 165.35$ ppm (Q), $\delta = 132.01$ ppm (P) and $\delta = 127.99$ ppm (O)] appeared after the transesterification. The carbonyl carbons next to the TEG at $\delta = 171.93$ ppm (G) and the adipate group at $\delta = 172.62$ ppm (L) appeared at the expected positions.
4.3.5.3. Michael Addition of Diethanolamine to (Acrylate)$_2$-TEG-(Acrylate)$_2$

Tetra-acrylated TEG ((acrylate)$_2$-TEG-(acrylate)$_2$, 0.96 g, 0.66 mmol) was reacted with DEA (0.34 g, 3.19 mmol, 0.31 mL, 1.2 eq. per acrylate group) in anhydrous DMSO (0.33 mL) in the presence of CALB (11 mg resin @ 20 wt% enzyme, $6.6 \times 10^{-5}$ mmol) (Figure 4.84).  

Figure 4.83. $^{13}$C NMR spectrum of the transesterification product (solvent: DMSO-d$_6$).
Figure 4.84. Michael addition of diethanolamine to (acrylate)$_2$-TEG-(acrylate)$_2$ in anhydrous DMSO in the presence of CALB. [DEA] = 4.98 mol/L, [(acrylate)$_2$-TEG-(acrylate)$_2$] = 1.03 mol/L; [CALB] = 1.0 $\times$ 10$^{-4}$ mol/L.

Figure 4.85 shows the NMR spectra of the Michael addition product. In the $^1$H NMR spectrum, the proton resonances corresponding to the vinylidene ($\delta$=6.18 ppm) and vinyl ($\delta$=6.32 ppm and $\delta$=5.97 ppm) protons of the acrylate groups disappeared after the Michael addition reaction. The methylene protons next to the hydroxyl groups appeared at $\delta$=3.60 ppm (t) and the integration ratio of the proton resonances attributed to the methylene next to the ester linkage at $\delta$=4.12 ppm (b), $\delta$=4.06 ppm (h) and $\delta$=4.24 ppm (r,s) appeared as (b+h) : (r,s) = 3:4 demonstrating successful functionalization.
Figure 4.85. NMR spectra of the (HO)$_4$-TEG-(OH)$_4$: (top) $^1$H NMR spectrum and (bottom) $^{13}$C NMR spectrum (solvent: DMSO-$d_6$).
The $^{13}\text{C}$ NMR spectrum shows that the carbonyl carbon of corresponding to adipic vinyl ester at $\delta=165.36$ ppm shifted downfield to $\delta=172.06$ ppm (P). The two carbons at the chain end are observed at $\delta=59.21$ ppm (H’) and $\delta=65.24$ ppm (G’). The carbon resonances corresponding to the acrylate group at $\delta = 132.01$ ppm and $\delta = 127.99$ ppm shifted upfield to $\delta = 50.06$ ppm (F’) and $\delta = 32.11$ ppm (E’), respectively. All other carbon signals correspond to the expected structure.

In conclusion, TEGs were successfully functionalized by the CALB-catalyzed transesterification of vinyl esters under solventless conditions. Furthermore, the eight hydroxyl-functional core [(HO)$_4$-TEG-(OH)$_4$] was prepared by the transesterification of 2-(acryloyloxy) vinyl adipate and the Michael addition of DEA in the presence of CALB.

4.4. Enzymatic Functionalization of PEGs

Previously, Dr. Sen from Professor Puskas’ research group investigated the functionalization of PEGs with vinyl esters using CALB-catalyzed reactions in organic solvents. The methacrylation and acrylation of PEGs with various molecular weights and molecular weight distributions in THF were quantitative in 24 hours. In this thesis PEGs were reacted with vinyl esters in the presence of CALB to create multifunctional chain ends that could be used as the building blocks for the targeted nanodevices and other biomedical applications. A tetra-hydroxyl functionalized PEG was prepared by CALB-catalyzed reactions using the same strategy of synthesis that produced (HO)$_2$-TEG-(OH)$_2$. 

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4.4.1. Transesterification of Vinyl Methacrylate with PEG

Solid PEGs ($M_n = 1000$ g/mol and 2000 g/mol) became liquid at 50 °C and were quantitatively functionalized by transesterification. PEG (0.50 g, 0.5 mmol, $M_n = 1000$ g/mol; $M_w/M_n = 1.08$) was reacted with VMA (0.56 g, 4.99 mmol, 0.60 mL, 5.0 eq. per OH in PEG) in the presence of CALB (50 mg resin @ 20 wt% enzyme, $3.0 \times 10^{-4}$ mmol, 10 wt% relative to PEG) under solventless conditions within 4 hours (Figure 4.86, 0.56 g yield, 100% conversion).

![Figure 4.86. Enzymatic transesterification of VMA with PEG ($M_n = 1000$ g/mol; $M_w/M_n = 1.08$) in the presence of CALB. [VMA] = 8.32 mol/L, [PEG] = 0.83 mol/L; [CALB] = $5.0 \times 10^{-4}$ mol/L.]

In the $^1$H NMR spectrum of Figure 4.87, the hydroxyl proton signals at $\delta=4.55$ ppm from the HO-PEG-OH disappeared and the peak corresponding to the methylene protons adjacent to the hydroxyl groups shifted downfield from $\delta=3.50$ to $\delta=4.22$ ppm (b) after the reaction. The new peaks corresponding to the vinyl [$\delta=6.07$ ppm (e) and $\delta=5.65$ ppm (e')] and methyl [$\delta=1.73$ ppm (f)] protons of the methacrylate group were observed at the expected positions.
Figure 4.87. NMR spectra of the methacrylation product of PEG ($M_n = 1000$ g/mol; $M_w/M_n = 1.08$): (top) $^1$H NMR spectrum and (bottom) $^{13}$C NMR spectrum (solvent: DMSO-$d_6$).

The $^{13}$C NMR spectrum of the methacrylation product also confirmed the structure of the polymer. The $^{13}$C NMR spectrum showed that the carbons connected to the hydroxyl groups at $\delta=60.13$ ppm in the HO-PEG-OH shifted downfield to $\delta=64.02$ ppm (B) and the carbon signals associated with the methacrylate group at $\delta = 17.95$ ppm (F) $\delta = 136.17$ ppm (F), $\delta = 126.20$ ppm (G) and $\delta = 166.68$ ppm (H) appeared after 4 hours reaction.

MALDI-ToF mass spectrometry was utilized for further confirmation of the chain end structure. A single product with the expected structure was observed as indicated in
Figure 4.88. For example, the peak at m/z 1101.61 corresponds to the sodium complex of the 21-mer of PEG dimethacrylate. The calculated monoisotopic mass for this ion is 1101.70 Da \([21 \times 44.03 \text{ (C}_2\text{H}_4\text{O repeat unit)} + 154.08 \text{ (C}_8\text{H}_{10}\text{O}_3 \text{ end groups)} + 22.99 \text{ (Na}^+)\)]. Within the series, the peaks were separated by 44 Da, corresponding to an ethylene glycol repeating unit. However, upon a closer examination, a minor distribution of peaks which differ from the main series by 16 m/z units were observed (see bottom of Figure 4.88). These were assigned to the K\(^+\) cationized PEG dimethacrylate as it is known that K\(^+\) contamination could occur during sample preparation\(^{174}\) and the mass difference between Na\(^+\) and K\(^+\) is 16 Da. Therefore, MALDI-ToF MS analysis confirmed that the conversion of HO-PEG-OH to PEG dimethacrylate was quantitative within 4 hours of reaction time under solventless conditions.

PEG with 2000 g/mol was also enzymatically methacrylated under the same conditions as above. \(^1\)H and \(^13\)C NMR spectroscopy and MALDI-ToF mass spectrometry confirmed a single product with the expected structure. In the MALDI-ToF mass spectrum shown in Figure 4.89, the peak at m/z 2026.26 corresponds to the sodium complex of the 42-mer of PEG dimethacrylate. The calculated monoisotopic mass for this ion is 2026.33 Da \([42\times44.03 \text{ (C}_2\text{H}_4\text{O repeat unit)} + 154.08 \text{ (C}_8\text{H}_{10}\text{O}_3 \text{ end groups)} + 22.99 \text{ (Na}^+)\)]. In the expanded spectrum, a minor series of peaks were distributed 16 m/z units above the main (sodiated) product series; the masses of these products agreed with those of potassiated PEG dimethacrylate.
Figure 4.88. MALDI-ToF mass spectrum of PEG dimethacrylate ($M_n=1000$ g/mol; $M_w/M_n = 1.08$).
Figure 4.89. MALDI-ToF mass spectrum of PEG dimethacrylate ($M_n=2000$ g/mol; $M_w/M_n = 1.20$).
4.4.2. Transesterification of Vinyl Crotonate with PEG

Crotonate groups were also successfully functionalized at the chain end of PEG (0.41 g, 0.41 mmol, $M_n = 1000$ g/mol; $M_w/M_n = 1.08$) by the transesterification of vinyl crotonate (0.43 g, 3.81 mmol, 0.46 mL, 5.0 eq. per OH in PEG) in the presence of CALB (42 mg resin @ 20 wt% enzyme, $2.5 \times 10^{-4}$ mmol, 10 wt% relative to PEG) in bulk for 4 hours reaction time (0.46 g yield, 100% conversion).

![Figure 4.90. Transesterification of vinyl crotonate with PEG ($M_n = 1000$ g/mol; $M_w/M_n = 1.08$) in the presence of CALB. [Vinyl crotonate] = 8.28 mol/L, [PEG] = 0.89 mol/L; [CALB] = $5.5 \times 10^{-4}$ mol/L.]

NMR spectroscopy and MALDI-ToF mass spectrometry confirmed the single transesterification product without any side products. Figure 4.91 shows the $^1$H and $^{13}$C NMR spectra of the transesterification product. The triplet peak of the methylene protons adjacent to hydroxyl groups shifted downfield from $\delta=3.50$ to $\delta=4.16$ ppm (c) after the reaction. New proton resonances attributed to the methyl [$\delta=1.85$ ppm (g)] and vinyl [$\delta=6.90$ ppm (f) and $\delta=5.90$ ppm (e)] of the crotonate group were observed at the expected positions. The $^{13}$C NMR spectrum showed that the carbons connected to the hydroxyl groups in the starting material shifted downfield to $\delta=63.12$ ppm (C) and the carbon signals associated with the crotonate group [$\delta=166.00$ ppm (D), $\delta=145.26$ ppm (E), $\delta=122.10$ ppm (F) and $\delta=17.55$ ppm (G)] appeared at the expected positions, confirming the successful functionalization.
Figure 4.91. NMR spectra of the crotonation product of PEG ($M_n = 1000$ g/mol; $M_w/M_n = 1.08$): (top) $^1$H NMR spectrum and (bottom) $^{13}$C NMR spectrum (solvent: DMSO-$d_6$).

The MALDI-ToF mass spectrum includes a peak at $m/z$ 1101.67 which corresponds to the sodium complex of the 22-mer of PEG dicrotonate (Figure 4.92). The calculated monoisotopic mass for this ion is 1145.73 Da [$22 \times 44.03$ (C$_2$H$_4$O repeat unit) + 154.08 (C$_8$H$_{10}$O$_3$ end groups) + 22.99 (Na$^+$)]. In the expanded spectrum, a minor series of peaks were distributed 16 $m/z$ units above the main (sodiated) product series; the masses of these products agreed with those of potassiated PEG dicrotonate (1161.68 Da). The calculated monoisotopic mass for the potassiated 44-mer is 1161.70 Da [$22 \times 44.03$ (C$_2$H$_4$O repeat unit) + 154.08 (C$_8$H$_{10}$O$_3$ end groups) + 38.96 (K$^+$)].
Figure 4.92. MALDI-ToF mass spectrum of PEG dicrotonate.
4.4.3. Transesterification of Vinyl Acrylate with PEG

Vinyl acrylate (VA, 0.32 g, 3.24 mmol, 0.34 mL, 5 eq. per OH) was reacted with PEG (0.32 g, 0.32 mmol, $M_n = 1000$ g/mol; $M_w/M_n = 1.08$) in the presence of CALB (35 mg resin @ 20 wt% enzyme, $2.1 \times 10^{-4}$ mmol) at 50 °C for 4 hours shown in Figure 4.93 (0.36 g yield, 100% conversion).

![Transesterification of VA with PEG](image)

Figure 4.93. Transesterification of VA with PEG ($M_n = 1000$ g/mol; $M_w/M_n = 1.08$) in the presence of CALB. [VA] = 9.53 mol/L, [PEG] = 0.94 mol/L; [CALB] = $6.2 \times 10^{-4}$ mol/L.

Figure 4.94 shows the $^1$H NMR spectrum of the PEG diacrylate. The peak corresponding to the methylene protons next to hydroxyl group shifted downfield from $\delta = 3.50$ to $\delta = 4.42$ ppm (c) after 4 hours. New resonances attributed to the vinylidene [$\delta = 6.20$ ppm (e)] and vinyl [$\delta = 6.32$ ppm (f) and $\delta = 5.97$ ppm (g)] protons appeared with the integration ratios of (c):(e):(f):(g) as 2:1:1:1. The $^{13}$C NMR spectrum of the transesterification product also confirmed the structure of the PEG diacrylate. The carbons connected to the hydroxyl groups in the starting material at $\delta = 60.13$ ppm shifted downfield to $\delta = 63.41$ ppm (C) and the carbon signals associated with the acrylate group at $\delta = 166.02$ ppm (D), $\delta = 131.86$ ppm (E) and $\delta = 128.55$ ppm (F) appeared after the acrylation.
Figure 4.94. NMR spectra of the acrylation product of PEG ($M_n = 1000$ g/mol; $M_w/M_n = 1.08$): (top) $^1$H NMR spectrum and (bottom) $^{13}$C NMR spectrum (solvent: DMSO-d$_6$).

Figure 4.95 shows MALDI-ToF mass spectrum of the transesterification product after 4 hours of reaction. The major distribution corresponds to PEG diacrylate. In the expanded spectrum, the representative peak at $m/z$ 1117.65 corresponds to the sodium complex of the 22-mer of PEG diacrylate; its calculated monoisotopic mass is [$22 \times 44.03$ (C$_2$H$_4$O repeat unit) + 126.11 (C$_6$H$_6$O$_3$ end groups) + 22.99 (Na$^+$)] 1117.73 Da. The peak at $m/z$ 1105.65, which belongs to a minor distribution differing from the main series by 12 $m/z$ units, corresponds to $\alpha$-acetate, $\omega$-acrylate functionalized PEG. The calculated monoisotopic mass for its 22-mer is 1105.75 Da [$22 \times 44.03$ (C$_2$H$_4$O repeat unit) + 126.11 (C$_6$H$_6$O$_3$ end groups) + 22.99 (Na$^+$)] 1105.75 Da.
unit) + 114.10 (C₅H₆O₃ end groups) + 22.99 (Na⁺)]. The acetate group most likely comes from vinyl acetate, an impurity in commercial vinyl acrylate.

Figure 4.95. MALDI-ToF mass spectrum of the transesterification product.
4.4.4. Synthesis of (HO)$_2$-PEG-(OH)$_2$

A four-functional PEG was synthesized using a similar synthetic strategy that was used in the synthesis of (HO)$_2$-TEG-(OH)$_2$ (section 4.1). The PEG diacrylate (1.01 g, 0.90 mmol) prepared as described in section 4.4.3 was reacted with diethanolamine DEA (0.20 g, 1.90 mmol, 0.18 mL, 1.05 eq. per acrylate group of PEG) in anhydrous DMSO (1.9 mL) at 50 °C for 24 hours via CALB (123 mg resin @ 20 wt% enzyme, $7.4 \times 10^{-4}$ mmol)-catalyzed Michael addition to yield (HO)$_2$-PEG-(OH)$_2$ (1.18 g yield, 98.3% conversion, Figure 4.96).

Figure 4.96. Michael addition of diethanolamine to PEG diacrylate in anhydrous DMSO in the presence of CALB. [DEA] = 0.91 mol/L, [PEG diacrylate] = 0.43 mol/L; [CALB] = $3.6 \times 10^{-4}$ mol/L.

Figure 4.97 shows the $^1$H and $^{13}$C NMR spectra of the (HO)$_2$-PEG-(OH)$_2$ product. The vinylidene [$\delta=6.18$ ppm] and vinyl [$\delta=6.32$ ppm and $\delta=5.97$ ppm] protons of the acrylate group disappeared and the peak corresponding to the protons of the hydroxyl groups appeared at $\delta=4.25$ ppm (g) after the Michael addition. The integral ratio of the chain end hydroxyl protons (g) to the methylene protons next to the ester linkage (b) was 1:1 confirming the expected structure. The $^{13}$C NMR spectrum of the Michael addition product also confirmed the structure. The carbons connected to the hydroxyl groups and the amine group at $\delta=59.69$ ppm (H) and $\delta=56.71$ ppm (I) appeared after the reaction and
the carbon resonances of the acrylate group shifted upfield to $\delta=50.32$ ppm (E) and $\delta=32.22$ ppm (F), respectively.

Figure 4.97. NMR spectra of Michael addition of DEA to PEG diacrylate: (top) $^1$H NMR spectrum and (bottom) $^{13}$C NMR spectrum (solvent: DMSO-d$_6$).

In summary, PEGs were successfully functionalized by the transesterification of vinyl esters using CALB. The presence of the chain end functional groups was confirmed by $^1$H NMR, $^{13}$C NMR and MALDI-ToF MS. A tetra-hydroxy functional PEG was prepared by the Michael addition of DEA with PEG diacrylate.
CHAPTER VI
CONCLUSIONS

This dissertation has investigated enzyme-catalyzed reactions to design and synthesize a multifunctional poly(ethylene glycol) (PEG) topology for addressing current problems of PAMAM dendrimer nanodevices.

In Section 4.1, we have shown that the four-functional core of the targeted device can very effectively be synthesized using enzyme catalysis. First, tetraethylene glycol (TEG) multifunctional core was synthesized by the transesterification of vinyl acrylate (VA) with TEG followed by Michael addition of diethanolamine to the resulting TEG diacrylate in the presence of CALB with/without the use of solvent. Specifically, the reactions occurred quantitatively under solventless conditions with 100% conversion. We confirmed that there were no byproducts using electrospray ionization mass spectrometry (ESI-MS). The functional PEG arms with fluorescein or folic acid (FA) were prepared using both traditional organic chemistry and enzyme-catalyzed reactions. Fluorescein isothiocyanate (FITC) was exclusively reacted with the amine group of H$_2$N-PEG-OH ($M_n$=1000 g/mol) in the presence of triethylamine via nucleophilic addition. Then, FITC-PEG-vinyl ester to be attached to the four-functional core via CALC-catalyzed transesterification was prepared by the transesterification of divinyl adipate (DVA) with FITC-PEG-OH in the presence of CALB.
For the FA conjugated PEG arm, DVA was first transesterified with BzPEG-OH ($M_n=1000$ g/mol) under solventless conditions in the presence of CALB. The BzPEG vinyl ester was then reacted with 12-bromo-1-dodecanol in the presence of CALB within 2 hours. Finally, FA was conjugated with BzPEG-Br using n-BuLi as our new conjugation method. We still need to complete the deprotection of the benzyl group followed by the transesterification of DVA with FA-PEG-OH. Then the functionalized arms need to be connected to the core.

In section 4.2, we performed fundamental studies in order to get better understanding of enzyme catalyzed transesterification and Michael addition reactions. First, the effect of reagent concentration was investigated in the transesterification of vinyl methacrylate (VMA) with 2-(hydroxyethyl) acrylate (2HEA) in the presence of CALB. In 4 hours of reaction time, complete conversion was achieved under solventless conditions with 2HEA concentration of 2.10 mol/L whereas 19% conversion was observed when the reaction was performed in tetrahydrofuran (THF) with 2HEA concentration of 0.10 mol/L. The effect of enzyme concentration in reactions with and without solvent was also studied. Quantitative conversion was achieved within 4 hours using CALB with $4.2 \times 10^{-3}$ mol in THF ([CALB]$_{\text{in THF}}=10^{-3}$ mol/L) and $4.2 \times 10^{-4}$ mol in bulk ([CALB]$_{\text{in bulk}}=2.0 \times 10^{-4}$ mol/L in bulk), respectively. There was no further rate increase with increased enzyme concentration over $4.2 \times 10^{-4}$ mol of CALB in bulk. It is worth mentioning that under solventless conditions the same catalyst reactivity could be reached when the enzyme concentration was reduced by an order of magnitude. CALB showed high chemoselectivity in the reaction of DVA with 11-mercapto-1-undecanol in
THF. DVA reacted only with the hydroxyl group of 11-mercaptop-1-undecanol while the thiol group remained intact. The regioselectivity was also studied by the CALB-catalyzed Michael addition of diethylamine to α-acrylate-ω-methacrylate and α-acrylate-ω-crotonate ethylene glycol which were prepared by the transesterification of VA, VMA and vinyl crotonate with 2HEA, respectively, under solventless conditions. Diethylamine exclusively reacted with the acrylate group of both compounds within 30 minutes. The enzyme regioselectivity most likely was the result of the steric hindrance around the vinyl groups at the α-terminus due to the presence of methyl group. The effect of steric hindrance was further confirmed in CALB-catalyzed Michael addition of diethanolamine to TEG dimethacrylate or TEG acrylate. The Michael addition of diethanolamine to TEG dimethacrylate only gave 12.1% conversion within 24 hours and no further progress was observed after 24 hours. In contrast, TEG diacrylate reacted completely with diethanolamine within 2 hours. The effect of DVA concentration on oligomerization was studied by the CALB-catalyzed transesterification of DVA with TEG and PEGs under solventless conditions. The vinyl ester functionalized PEGs were successfully prepared by the CALB-catalyzed transesterification of DVA with PEGs. Increasing the DVA concentration decreased the extent of coupling. At higher PEG MWs no coupling was observed.

In line with these fundamental studies, we discussed the enzymatic functionalization of TEGs and PEGs in section 4.3 and 4.4, respectively. TEGs and BzTEGs were quantitatively transesterified with VMA and vinyl crotonate in the presence of CALB under solventless conditions. Eight hydroxyl-functional TEG [(HO)₄–
TEG–(OH)$_4$ was also prepared by the CALB-catalyzed transesterification using 2-(acryloyloxy)ethyl adipate as a vinyl-acrylate functionalized linker which was prepared by the transesterification of DVA with 2HEA, followed by Michael addition of diethanolamine to the tetra-acrylated TEG. Moreover, PEGs were also successfully functionalized by the enzymatic transesterification using vinyl esters such as VMA, vinyl crotonate and VA under solventless conditions within 4 hours. PEG diacrylate was further functionalized by the Michael addition of DEA, resulting in a tetra-hydroxy functional PEG [(HO)$_2$-PEG-(OH)$_2$].

In summary, this dissertation has provided new approaches to design and synthesize multifunctional polymeric structures using enzymatic catalysis for biomedical applications. However, a definite need for improved understanding of enzymatic reactions remains a challenge.
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APPENDIX: CANCER DRUGS

A. Methotrexate

Methotrexate (MTX) is a widely used chemotherapeutic drug for the treatment of a variety of cancers.\textsuperscript{111, 176, 177} The chemical structure of MTX is very similar to FA. MTX as an antimetabolite and antifolate drug is acting by inhibiting the metabolism of folic acid. The higher doses of MTX often used in cancer chemotherapy can cause toxic effects to the rapidly dividing cells of bone marrow and gastrointestinal mucosa. MTX has been encapsulated into generations 3 and 4 PAMAM dendrimers, which had PEG\textsubscript{550} and PEG\textsubscript{2000} monomethyl ether chains conjugated to their surfaces to modify bioavailability and toxicity. The encapsulation efficiency was dependent on PEG chain length and size of the dendrimer, with the highest encapsulation efficiency of 26 MTX molecules per dendrimer found for PAMAM G4-PEG2000.\textsuperscript{65} In order to chemically conjugate the MTX, the remaining amino groups on the surface have to be conjugated with glycidol groups.

B. Paclitaxel

Paclitaxel (Taxol) is an anticancer drug as a mitotic inhibitor which is used in chemotherapy to treat patients with lung, ovarian, breast, and head and neck cancers.\textsuperscript{178, 179}
The drug works by interfering with normal microtubule growth during cell division, which especially affects fast growing cancer cells. In order to enhance its poor water solubility and specificity to cancer cells, Paclitaxel has been conjugated to polymeric macromolecules.\textsuperscript{180, 181}

Encapsulation of Taxol into polyglycerol dendrimers resulted in a 400-fold improved water solubility compared to the pure drug.\textsuperscript{182} Based on the conjugation of Taxol into multifunctional polymeric drug delivery carriers, undesirable side effects by the toxic Taxol were lessened or eliminated by targeting over-expressed genes or receptors on cancer cells.\textsuperscript{179, 183, 184}

C. 5-Fluorouracil

5-Fluorouracil (5-FU) is a pyrimidine analogue that belongs to the family of drugs called antimetabolites. Some of its principal uses are in colorectal and pancreatic cancers, in which it has been the established form of chemotherapy for decades. As a pyrimidine analogue, 5-FU is transformed inside the cell into different cytotoxic metabolites, which are then incorporated into DNA and RNA, inducing cell cycle arrest and apoptosis by inhibiting the cell’s ability to synthesize DNA. A conjugate between PAMAM G4 dendrimer and PEG5000 chains has been utilized in order to improve the solubility of fluorouracil. Drug loading into this PEGylated PAMAM was enhanced 12-fold compared to the respective non-PEGylated dendrimer, while release rates were enhanced 6-fold, allowing sustained release of 5-fluorouracil over a period of six days from PEGylated PAMAM G4.\textsuperscript{185}