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SYNTHESIS OF CORE-MULTISHELL ARCHITECTURES BASED ON EPOXIDE AND GLYCIDOL MONOMERS AS DRUG DELIVERY SYSTEMS

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The project described in this thesis was carried out in the research group of Prof. Dr. Rainer Haag, in the period from April 2015 to the end of August 2015 at the Institute of Chemistry and Biochemistry of the Freie Universität Berlin.

1. Reviewer: Prof. Dr. Rainer Haag, Free University of Berlin
2. Reviewer: Dr. Florian Paulus, Free University of Berlin
3. Reviewer: Prof. Dr. Michela Signoretto, University Ca' Foscari Venice

I certify that all the work presented in this thesis is my own original work based on the research I performed during the period of my Erasmus in the research group of Prof. Dr. Rainer Haag by using only the means and the source materials as noted therein.

Magda Ferraro
October 2015

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Abstract

A library of different epoxide based core-multishell architectures (CMS) was synthesized.

Based on an anionic polymerisation process, the polymers consist of a complete polyether backbone. Using a two-step process, it was possible to create two different shells on a hyperbranched polyglycerol core (hPG).

Epoxide derivatized monomers were utilised as building blocks for the inner shell, while the outer shell was made up from ethoxyethylglycidyl ether (EEGE).

Both the shells were developed through anionic ring opening polymerisation, using a “grafting-from” approach from the peripheral hydroxyl groups of the core.

In order to investigate the influence of monomer on the final CMS, propylene oxide and butylene oxide were compared and six products were formed, three made of each monomer.

Attention was further pointed on the impact of repeating units composing the inner shell of the polymer. The molar ratio between the hydroxyl groups present on the hPG core and the monomer was investigated. In particular, the ratios 1:5, 1:10 and 1:20 were studied. The entire library was characterized using Gel Permeation Cromatography (GPC), Nuclear Magnetic Resonance (NMR) and Dinamic Light Scattering (DLS).

The performance of each carrier was investigated by the transport capacity of model dyes. Tests were made by encapsulation of Nile red and pyrene and quantified by UV/Vis spectroscopy.

Finally, transport capacity of Dexamethasone, a drug used in treatment of skin diseases, was also tested; the performance was compared with similar CMS, used as benchmark and quantifications were made by High Performance Liquid Cromatography (HPLC) analysis.

Summary

1. Introduction

Several decades ago, the study of drug delivery and its enhancement has reached the interest of many researchers. In fact, many efforts are made to create systems able to reach the target avoiding side effects.

Regarding the delivery of drugs, the main question on which researcher have to focus is their formulation. Often drugs are small molecules, which require a carrier to transport and protect them from an immune response and degradation.[1] Therefore, the efforts are direct to the creation of systems capable, e.g., of transporting the drug to the desire target avoiding accumulation in healthy tissues and deterioration. Moreover, all these systems should be biodegradable and biocompatible.[2]

1.1 Dendrimers and hyperbranched polymers

In order to reach these objectives, work was made to develop systems which mimic the natural ones, such as liposomes and micelles. These phospholipidic vesicles are appreciated as they permit to encapsulate both hydrophilic and hydrophobic drugs.[3] However, they are instable to stress as temperature and dilution,[4] and present a reduced matrix compatibility.[5]

To overcome this problem, more specific polymers were investigated.

At first, dendrimers gained much attention and interest. These synthetic macromolecules possess highly branched arms, three dimensional shape and globular size.[6] Moreover, they exhibit unique characteristics, such as monodispersity and biocompatibility, but also chemical stability and inertness.[7] In general, the monomers are attached on a multifunctional core.[8]

These aspects make the polymers close to natural systems as, despite the functionalization, they present the typical features of liposomes and

micelles, but they also results more stable. Furthermore, they possess attractive characteristics, as size and degree of branching, which can be tuned during the synthesis. [9]

There are numerous techniques which can be used for synthesising these molecules, e.g. the divergent and convergent methods.[10] In the first method, a focal point is obtained through coupling with the branches; subsequently dendrons are achieved through divergent core anchoring.

It can be seen as a structure growing from inside to outside.

The second approach requires, on the other hand, to activate the functional groups of the surface and then to add the monomers.[8] In this process, single structure are therefore prepared and then assembled together. It represents the opposite way to obtain the same structure.

Figure 1 represents a scheme of the two methods.

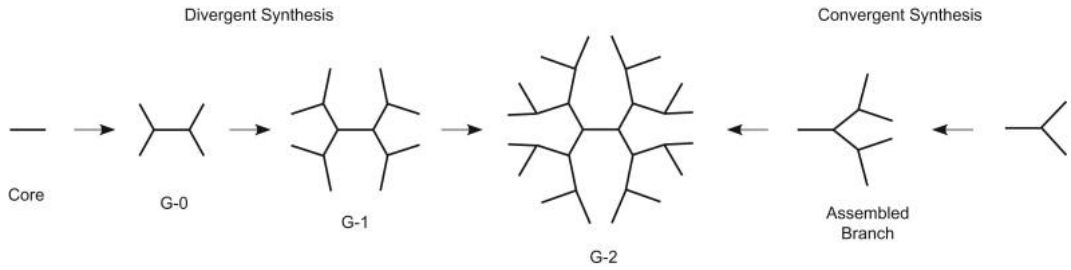

Fig. 1: Divergent and Convergent approaches[11]

The convergent synthesis permits to control the final structure better than the divergent technique; however, the second one is more appropriate for large scale applications.[12] In figure 2 a schematic representation of a dendrimer is reported.

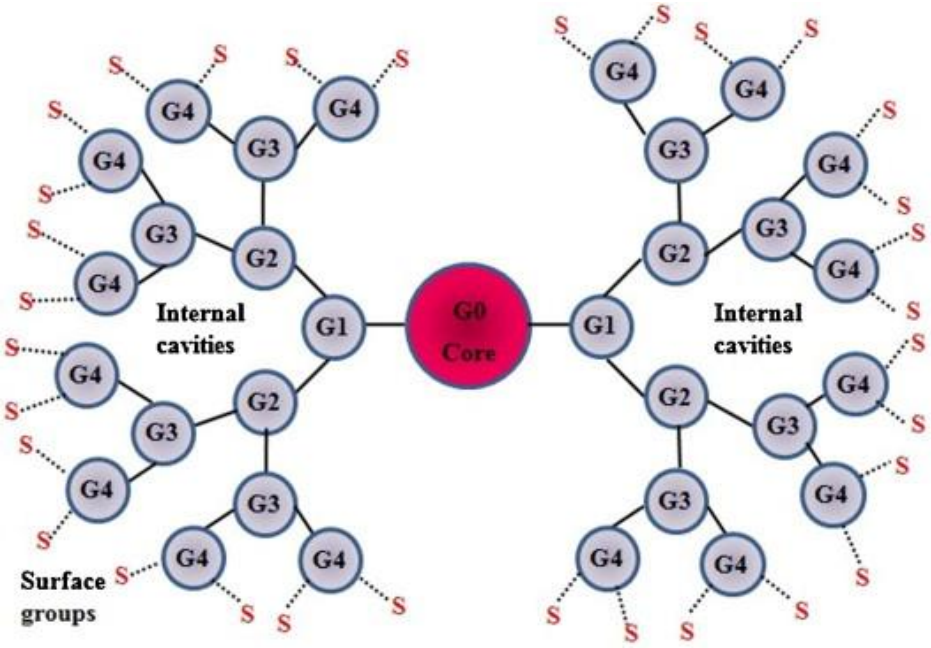

Fig. 2: General structure of a dendrimer [8]

Unfortunately, the synthesis of dendrimers represents a complicated, time consuming and expensive route; which mainly limits their application.[13]
Today there's another class of polymers which is gaining much more consideration: the hyperbranched polymers.
These structures are really close to dendrimers; they are composed of a backbone which can possesses different functional groups at the end of each branch.[14] Furthermore, the synthesis of dendrimers results tedious while hyperbranched polymers can be obtained through an easier synthetic path way.[15]
The major problem related to these molecules deals with their structure: in fact, contrary to dendrons, their production bring to a broad distribution of molecular weights.[16]
These polymers can be obtained using different kinds of polymerisation.[17]
At first, two method were mainly used: the polycondensation of an AB monomer and the self-condensing vinyl polymerisation.[18] Even if both the

syntheses lead to the desired target, they are fatiguing and the polydispersity index results broad.

An interesting synthesis is, on the other hand, represented by the controlled ring-opening multibranched polymerisation (ROMB), which is a relatively easy technique.[19] It consist in the polymerisation of a latent AB_m monomer.[20]

It is possible to obtain polymers with a narrow polydispersity by controlling the reaction conditions, e.g., the rate of monomer addition.[21]

Through this method is possible to achieve polymers which present molecular weight and polydispersity index in the desired range. Moreover, this class of polymers exhibit enhanced qualities, which also permit to use them as scaffolds.[4]

1.2 Hyperbranched Polyglycerol

Regarding the construction of the polyether based architectures, one of the favourite monomers is represented by glycidol, depicted in figure 3.

This molecule is a highly reactive epoxide, commercially available. As required, it is a latent AB_2 monomer and through its polymerisation a polyether chain presenting numerous hydroxyl groups is obtained.

The mechanism of polymerisation is reported in figure 3.

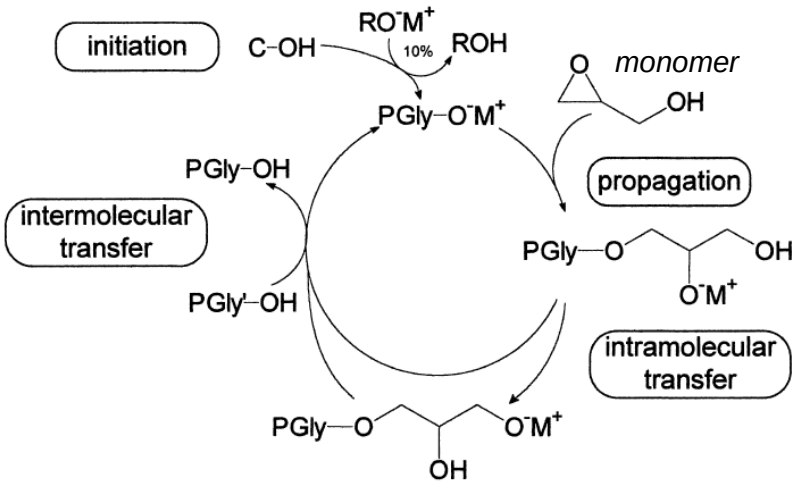

Fig. 3: Mechanism of the polymerisation of glycidol[19]

The initiation step forecasts the protonation of the core and the formation of the active alkoxide. In the propagation step, the monomer is added and additional hydroxyl groups are obtained through polymerisation. Then, intramolecular and intermolecular proton transfer occurs, so that all the hydroxyl species remain potentially reactive and a branched structure can be obtained.

Another important feature of this polymer is its similarity with polyethylene glycol (PEG),[22] a highly compatible polyether already approved by Food and Drug Administration (FDA) for several uses.[23]

Furthermore, as the hydroxyl groups present on the scaffold are potentially active, it is possible to functionalise the system and use it in different fields. Figure 4 reports some examples of its versatility.

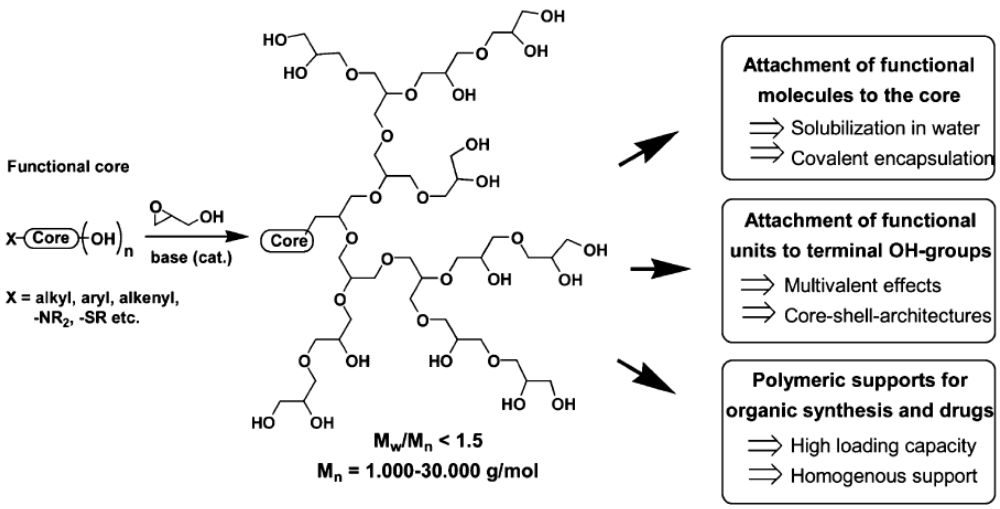

Fig. 4: Structure of hPG and possible functionalisation methods[24]

1.3 Core-Multishell Architectures

Thanks to their interesting properties, hyperbranched polymers can also be utilized to build core-multishell architectures (CMS), which are composed of a polar core, a non polar inner shell and a hydrophilic outer shell.

A schematic representation of CMS structure can be seen in figure 5.

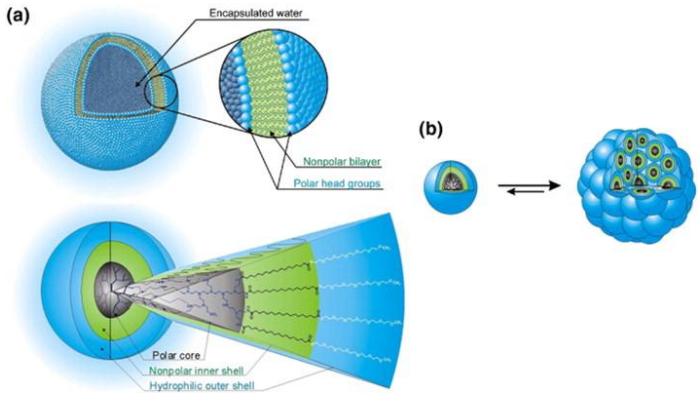

Fig. 5: Schematic representation of a CMS[22]

Core-multishell architectures exhibit an arrangement which is really close to natural liposomes. As previously explained, these natural vesicles find already biomedical applications. Liposomes are formed by self-assembly of phospholipidic layers; an aqueous medium can be observed both in the vesicle and surrounding it.[25] Thanks to this structure, these molecules are able to host non-polar compounds in their bilayer; moreover, also hydrophilic molecules could be entrapped in the exterior layer.

However, liposomes are susceptible to natural trigger such as temperature and concentration, so they do not represent a stabile supramolecular structure.

In particular, the critical micelle concentration (CMC) represents a fundamental parameter regarding the stability of an amphiphilic structure.

The CMC represent the concentration at which surfactants aggregate and form micelles.[26]

This behaviour is directly related to the free energy of the system: as the amount of surfactant increases the free energy decreases, so that at the CMC the energy reach its minimum and does not change anymore. By this way, a stabile mean is obtained.[27]

Nevertheless, the concentration required to reach the CMC is specific for each amphiphil and depending of various aspects, therefore it represents a limiting aspect of natural systems.

The core-multishell architectures possesses an own parameter which is similar to CMC: the critical aggregation concentration (CAC). It represents the concentration at which nanocarriers start to form aggregates.[23]

This parameter can strongly influence the stability of the system and the way that drug delivery is performed.

As already explained, core-multishell architectures mimic liposomes and are able to encapsulate various type of drug or molecules and to transport them both through aqueous and organic mediums.[5]

In order to obtain structures which possess areas of different polarity, various synthetic routes were developed. A turning point in this field is represented by the work of Radowsky.[25] It was demonstrated that the architectures were able to transport both hydrophilic and hydrophobic drug, adapting themselves to the environment, and therefore they were promising for various applications.

From that moment, various studies were made on structure, on its modification and their applications, in order to obtain better systems.[28–30]

Another important aspect related to CMS is that these macromolecules can be utilised for passive targeting. In fact, they can accumulate in solid tumour tissue.

Generally, molecules which possess low molecular weight can access to cell through endothelia tissue. This behaviour is not shown by macromolecules. Tumour tissue, however, results irregular, the endothelial tissue is often porous and therefore tends to absorb all the molecules as they were nutrients.[31] Because of the different biochemical and physiological characteristics, this situation is limited to damaged tissue. This behaviour is known as the EPR effect (Enhanced Permeability and Retention). Because of this property, the carriers containing the drug will reach the desired target and will not be rapidly eliminated by the kidneys.[22]

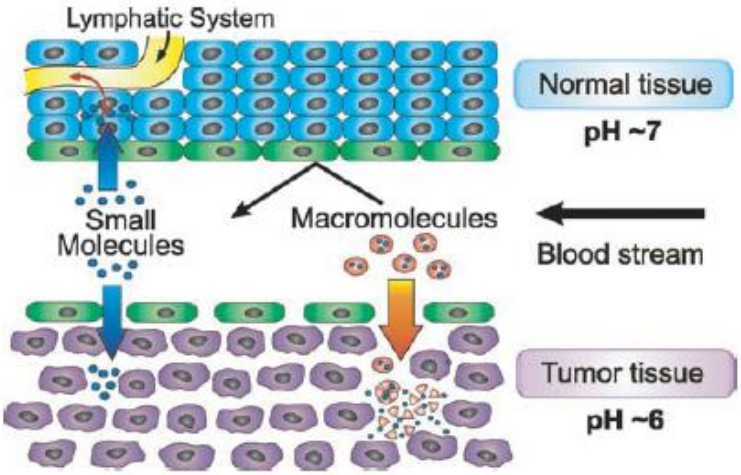

Fig. 6: Representation of a healthy tissue compared to the tumour one.[1]

Another possibility in cancer treatment is represented by the active targeting. Differently from the passive method, in this case the operation is based on the affinity between a receptor and the target site.

In figure 7 it is possible to observe a comparison between the two methods.

As previously described, passive targeting leads to an accumulation of loaded drug in ill tissue, meanwhile free molecules can enter and escape from it.

On the other hand, thank to ligands possessed by the molecules, in active targeting a bond with tumour is created, so that nanocarriers are hold in the target point.

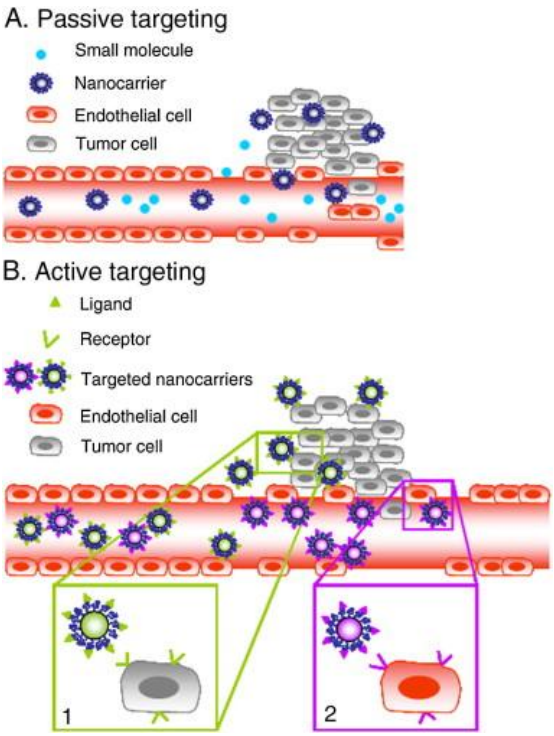

Fig. 7: Passive targeting vs active targeting[32]

The active targeting, due to the required specific connections, results more suitable for those systems which cannot operate by passive targeting.

1.4 Formulation and dermal transport

Drug delivery can be carried out through different ways: oral administration, mucosal, dermal, intravenous, intramuscular and rectal.
Every administration pathway presents some strong points and some disadvantages, which are summarised in figure 8.

Table 4.1 Advantages and disadvantages of different routes of medicine administration[a]

Route	Advantages	Disadvantages
Oral	Cheap, easy, no special equipment. Acceptable to most people. Suitable for self-medication.	May be compromised by irritant effects/ presence of food. Enzyme action may limit effectiveness.
Sublingual	Drug absorption through buccal or sublingual mucosa avoids gut enzymes. Rapid action.	Taste of drug may be a problem.
Transdermal	Easy to use. Long action can be achieved. Avoids adverse effects of gastrointestinal tract enzymes.	Relatively high cost. Drug may build up in skin so that action continues when patch removed.
Inhalation	Rapid action (inhaled anaesthetics). Limits systemic absorption. Avoids gut enzymes.	Needs specialised drug delivery system. Loss of dose – patient swallows most of drug. Technique needs to be taught.
Intranasal	Similar to inhalation.	May irritate nasal mucosa. Needs special drug delivery system. Absorption may vary.
Subcutaneous	Rapid absorption. Bypasses gastrointestinal tract. Patients may be taught to use this method.	Absorption may be too rapid.
Intramuscular	Good absorption. Bypasses gastrointestinal tract.	Local irritancy. May be painful. Hazard of nerve damage. Skill involved.
Intravenous	Rapid action can control rate of administration. Suitable for large volumes and drugs that would be irritant intramuscularly.	Relatively high cost. Skills involved. Extravasation risk. Specialist drug delivery system needed.
Rectal	Suitable for drugs that may irritate the upper gut. Fairly rapid action.	May not be acceptable to some people. Variable absorption.

[a]It is important to note that many factors affect drug absorption. The chemical properties of the drug and physiological variables (e.g. blood flow) all influence the rate at which a drug is absorbed.

Fig. 8: General way of drug administration[33]

In general, all these methods suffer of a reduced targeting, due to natural barriers which formulations encounter on their path, such as blood and its constituents.

The fastest way to bring a drug in our circulatory system is assured by intravenous administration: blood will flow first in heart and lungs, and then will be pumped through the entire body. Moreover, large volumes can be administered. In this way, however, a lot of the injected means will reach

healthy instead of the diseased tissue; furthermore, it represents an expensive way, which requires specific equipment.

Generally, oral administration is more tolerated by patients, as can be self-administered and is generally an economic therapeutic choice. However, the action of drugs can be reduced by interactions with food, drink and enzymes present in our body.

Focusing on the dermal application, it is one of the generally preferred by the patient, because it is non-invasive. Furthermore, risk of side effects, inflammations and infection are reduced.[34]

Moreover, this route presents some advantages such as bypass of hepatic metabolism, simplification of dosing and use.[35]

Due to this aspect, several studies have been made regarding skin structure and the permeation of polymers through it.

In general, polymers owning high biocompatibility represent an interesting means for in vivo applications.[36] Tailoring their characteristic such as rate of degradation, interaction with body components, size and shape, it is possible to employ them in various field of biomedicine.

Regarding the cutaneous application, polymers can be employed, e.g., for host guest applications.

Skin represents the first barrier which protects our cell and tissue. It is formed by numerous layers, which differ in structure and composition.[37][38]

The dermis is a hydrophilic stratum located in under the epidermis, which is in turn divided in a hydrophilic layer and a hydrophobic one, the stratum corneum[39] (see figure 9).

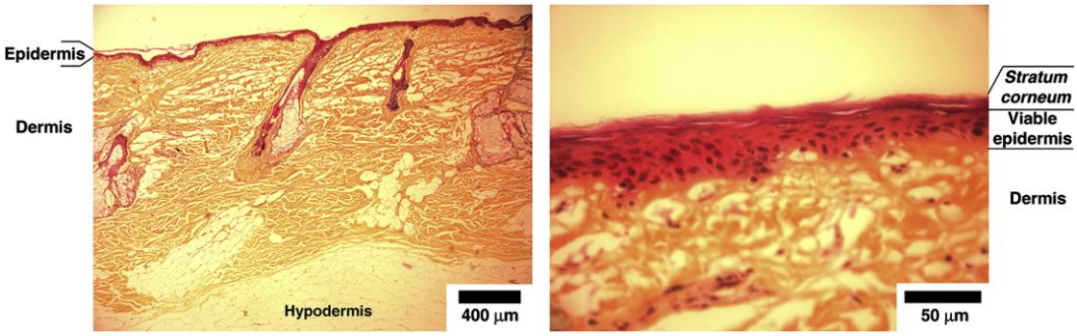

Fig. 9: Section of skin at optical microscope[39]

The dermal adsorption is composed of three steps: penetration, permeation and resorption.[39] This means that at first the molecules should be able to enter the skin; then, they should move from a layer to another; finally they should be assimilated. A drug should therefore be capable of proceeding through these ambient and reach the deepest layer in order to be absorbed; however, due to the physical and chemical characteristic, a nude molecule will not be able to interact successfully with all these layers.

In order to overcome these problems, CMS represent a system with great potential to deliver drugs by dermal applications.

2. Scientific Goal

As previously explained, the study of drug delivery is a central point in current research topics of many scientists worldwide. Focusing on dermal applications, the development of new structures capable to penetrate the different layers of skin is an interesting approach to improve the therapeutic capability of a drug.

The present work was inspired by a successfully developed CMS which among others is able to transport short molecules into the skin.

Nevertheless, the synthesis present some disadvantages, as a pathway divided in various steps.[23]

The aim of the work was to synthesise CMS using different epoxides. In fact, these highly reactive molecules are expected to react greatly with the polyglycerol core and to lead to a faster synthesis.

The project forecasted to build a hydrophobic shell on the core, subsequently followed by growing a hydrophilic shell on this architecture.

Two epoxides were used, in order to compare how the side chain influences the characteristics of the carrier. Propylene oxide and butylene oxide were chosen as starting materials of the hydrophobic shell. The outer shell was built using ethoxyethylglycidyl ether (EEGE).

Moreover, it is interesting to investigate other parameters which can influence the behaviour of the carrier. It was chosen to synthesise various samples, changing the characteristics of the inner shell.

The attention was focused on the molar ratio between the hydroxyl groups of the core and the epoxide monomer. A library of six polymers was obtained choosing the ratios 1:5, 1:10 and 1:20.

The loading capacity was investigated using model dyes Nile red and pyrene in order to compare the performance of the different systems.

Finally, the encapsulation properties of Dexamethasone, a drug used in skin diseases, was investigated. Comparison was made with CMS successfully encapsulating DMX used as model.

3. Results and discussion

3.1 Introduction

3.1.1 Anionic ring opening polymerisation

The ring opening polymerisation (ROP) is an interesting technique which consent to synthesise polymers with controlled characteristics. [40]
In order to obtain the desired polymer, it is fundamental that the reaction is thermodynamically and kinetically allowed.[41] In fact, the equilibrium of the reaction should be shifted to the products and the reaction should occur in typical polymerization time.
According to different mechanisms, it is possible to carry on different types of ROP. The most common methods are radical ROP, cationic ROP and anionic ROP.[40] In relation to this work, the best method is represented by the anionic ring opening polymerisation (AROP).
This kind of polymerisation is particularly appreciated as it allows to synthesise polymers with desired structure and controlled molecular weight.[42] Moreover, it represents a living polymerisation; therefore, the anionic centres remain active and no termination of polymerisation is expected. As all the growing chains develop in the same way, it is possible to achieve a narrow polydispersity index.
The mechanism of the anionic polymerisation is presented in figure 10.

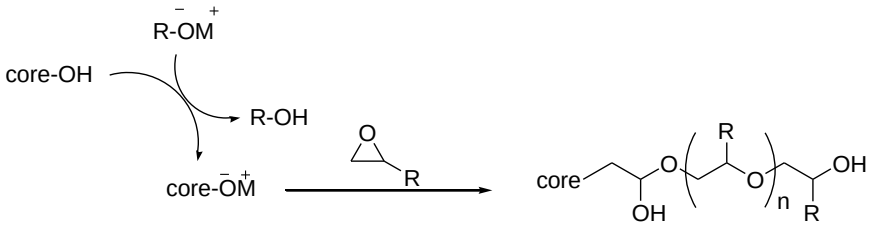

Fig.10: AROP of propylene oxide on a core possessing OH terminal groups

In the first step, the metal deprotonates and activates the core, allowing the second step to happen. In fact, the hydroxyl groups of the core act as initiators of the reaction. After that, a nucleophilic attack on the ring occurs, leading to the final structure.
The reaction continues as long as there is free monomer and no termination step occurs. The same mechanism is used for growing the outer shell; the ether is reacted with the terminal groups of this molecule.

3.1.2 Side reactions

In presence of heterocyclic molecules not symmetrically substituted, the ring opening polymerisation can proceed through two ways. A scheme of the mechanism is presented in figure 11.

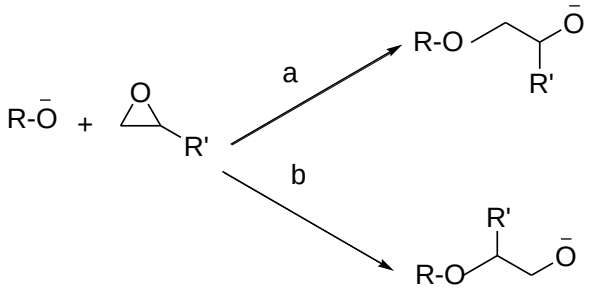

Fig.11: Schematic representation of possible paths.

The nucleophilic attack (S_N2) should happen on the less substituted carbon, leading to the desired product. However, it is also possible that the polymerisation starts on the other carbon, building the structure shown by path “b”. Even if both reactions are possible, it was demonstrated that the first pathway “a” represent the preferential way.[40]
Depending on the R’ substituent on the ring, different products can be obtained. It can act as initiating specie, as previously described, or it can be used to functionalise the product. Moreover, as R’ can provoke steric hindrance, the formation of a product despite his isomer can be favoured.
Moreover, in presence of alcohol, an exchange reaction can happen: in presence of metal alkoxides the following exchange is possible.[43]

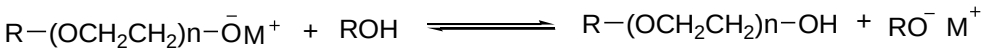

Fig.12: Exchange reaction

This kind of reactions can also involve two growing chains.
The main effects of side reactions are represented by reduction of molecular weight and growth of polydispersity.

3.1.3 “Grafting-from” technique

The modification of polymer surfaces is a widely used method to get new structures. The “grafting” techniques represent a common strategy to reach this goal. Indeed, these methods present some advantages, as they permit to introduce various type of functionalities and lead to stable products.[44]
In general, the most common ways to functionalise polymers are the “grafting-to” and the “grafting-from” process.

The first one consists of the reaction between complementary terminal groups which are located at the end of the chains. However, it is not possible to bind a huge amount of polymer. Indeed, it is necessary that the branching chain passes the physical barrier represented by the polymer on which it should be grafted and therefore only a little amount can reach the surface.[45]

The “grafting-from” technique represents, on the other hand, an easier process: a covalent bond through surface and initiator is made, then the polymer is grown directly on the solid.[46]

At first, it is necessary to introduce the initiator on the selected surface, subsequently a polymerisation is conducted. The method is compatible with almost all the polymerisation techniques.

In figure 13 a schematic representation of the techniques is shown.

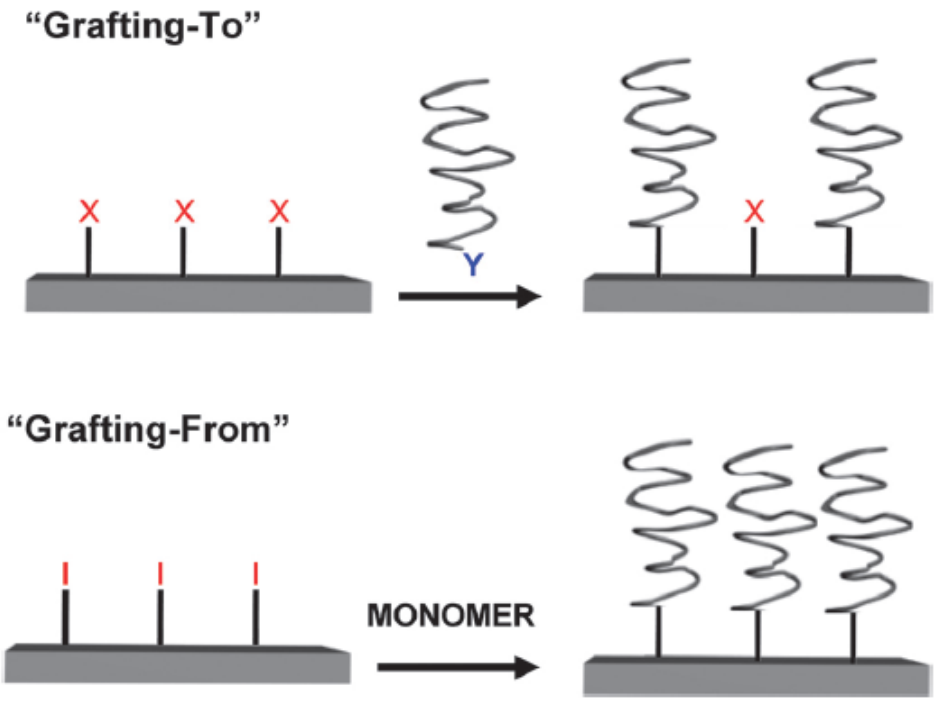

Fig. 13: Example of grafting to and grafting from[47]

3.1.4 Choice of the monomer

The purpose of this work was to create a new structure which possesses characteristics similar to those of CMS model described in 1.3. Therefore, it was necessary to build a hydrophobic and a hydrophilic shell.

Regarding the inner shell, the attention was focused on epoxides derivates. In fact, these molecules are extremely reactive because of the ring strain and are consequently attended to react abundantly.[48]

Moreover, the polymerisation of epoxides leads to a non polar chain, which possesses terminal hydroxyl groups, which can be exploited to react in a new polymerisation.

Propylene oxide and butylene oxide were chosen as they are relatively cheap starting materials. Moreover, these epoxides present an acceptable compromise between safety and reactivity.

Concerning the outer shell, ethoxyethylglycidyl ether was the selected monomer. Also this molecule possesses a heterocyclic part, which can easily react with the hydroxyl group formed on the polymer previously obtained, through the said ROP reaction.

However, the polymerisation of this ether does not yield the desired target, as a non polar protected polymer has grown. In order to reach the goal, it suffices to deprotect the product in acidic medium, as EEGE is strongly labile in this environment. This process leads to a linear polyglycerol.

3.2 Synthesis and characterization

3.2.1 Previous works on CMS

The original synthesis of CMS is a multistep pathway, according to Radowski.[25] A schematic representation of the process is shown in the following figure.

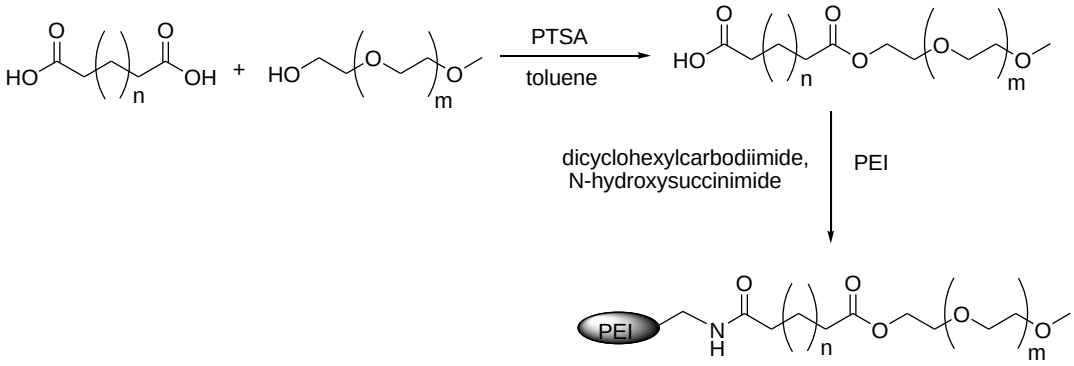

Figure 14: Original synthesis of CMS.

At first, a dicarboxilic acid (C_6, C_{12} or C_{18}) is reacted with monomethyl poly(ethylene glycol) (mPEG); the reaction is made in toluene containing p-toluenesulfonic acid. After purification, an activation step is required. In particular, dicyclohexylcarbodiimide and N-hydroxysuccinimide are added to the mPEG-acid, in order to create a better leaving group and facilitate the following reaction. After that, the product is reacted with the hyperbranched poly(ethylene imine) (PEI). Finally, the product is purified by dialysis. However, even if PEI is cheap and commercially available, a major disadvantage in its use is related to the fact that at higher concentration and molecular weight it suffers of toxicity.

Arising from this work, additional studies were made, leading, e.g., to a synthesis which employs an hyperbranched polyglycerolamine core.[23] However, the reaction still results complicated and time consuming.[34]

3.2.2 Synthesis of CMS from epoxides

In order to develop an easier synthesis to obtain a product which maintains similar characteristics, concentration was focused on new monomers.

Figure 15 presents the synthesis of the inner shell used for both the monomers.

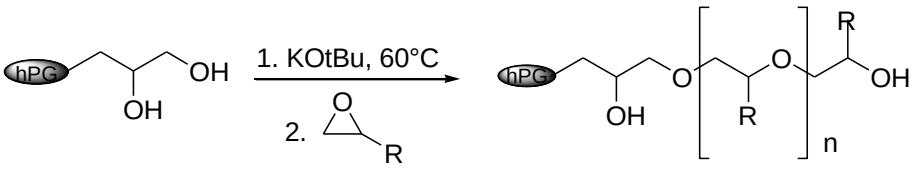

Fig.15: Synthesis using a general epoxide

First, a shell composed of aliphatic chains was obtained on a hyperbranched polyglycerol core (hPG) of 9.9 kDa.

The core, dissolved in methanol, is initially put under inert conditions; potassium *tert*-butoxide, corresponding to 5% mmol of initiator, is added to activate the core and system is heated to 60°C. Methanol is evaporated and the solvent (NMP) added, subsequently the temperature is increased to 95°C. As the core is completely dissolved, the required amount of monomer necessary to obtain the desired degree of polymerization (DP_n) is added.

The epoxide is slowly added over 4 h using a syringe pump and the system is kept under stirring for 18h. The product is cooled down and the solvent is evaporated under reduced pressure. Finally, purification through dialysis is executed.

The second step corresponds to the grafting from poly(ethoxyethylglycidyl) ether (PEEGE) to the previous system. The same reaction path is applied. The reaction is shown in figure 16.

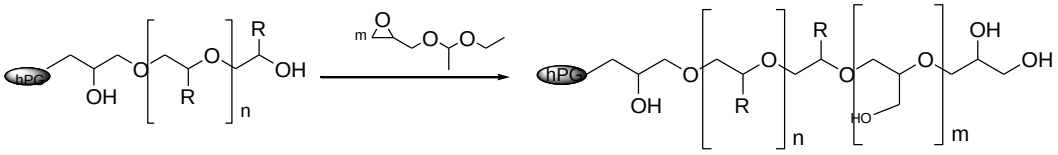

Fig.16: Synthesis of outer shell

The product is put under inert conditions; potassium *tert*-butoxide, corresponding to 5% mmol of initiator, is added and system is heated to 60°C. Methanol is evaporated and the solvent (NMP) added, then temperature is increased to 95°C. As the system is completely dissolved, the required amount of monomer, necessary to obtain a DP_n of 5, is added drop wise over 4 h and system is kept under stirring for 18h. The product is cooled down and the solvent is evaporated under reduced pressure. Dialysis is performed to purify the product.

3.2.3 Determination of repeating units and polydispersity index

As previously explained, it was decided to investigate the influence of the chain length on the final product.

It was in fact expected that increasing the size of the shells would lead to a distinct behavior of the polymers. In order to obtain this aim, it was necessary to determinate how to build chains of different length.

In general, to define the degree of polymerization (DP_n) of a polymer, the following equation is used:

$$DP_n = \frac{[M]}{[I]}$$

[M] is the molar concentration of monomer, while [I] represents the molar concentration of initiator.

The initiator of the reaction is represented by the hPG core. It was possible to determinate that 1 g of hPG of 9.9 kDa possesses 134 mmol of

hydroxyl groups. Starting from this assumption, it was necessary to choose the desired DP_n.

First of all, it was decided to change the DP_n of only the inner shell: the DP_n of the outer shell was kept constant, so that it was possible to study the influence on polymer performance depending only on one parameter. The second step was the choice of the desired DP_n. The decision was to investigate three ratios between the molar amount of hydroxyl groups and the molar concentration of monomer. Therefore the DP_n of 5, 10 and 20 were chosen. Regarding the outer shell, it was establish to maintain a DP_n of 5.

Once the polymers were obtained and purified, the entire library was characterized through gel permeation chromatography (GPC).

This technique consents to determinate some characteristic parameters of polymers, such as number average molecular weight (M_n) and weight average molecular weight (M_w).

Using these elements, it is possible to estimate the obtained repeating units. In order to evaluate the chain length, the following equations were used.

The degree of polymerization corresponds to the ratio of number average molecular weight (M_n) and molecular weight of the monomer (M_0).

$$DP_n = \frac{M_n}{M_0}$$

M_n = Number Average Molecular Weight

M_0 = Molecular weight of the monomer

The number of repeating units can then be obtained as the ratio between the DP_n and the molar amount of hydroxyl groups belonging to the core.

$$r.u. = \frac{DP_n}{134}$$

r.u. = repeating units

Moreover, the same parameters permit to calculate the polydispersity index (PDI), which is a useful parameter to understand the weight distribution of polymers.

$$PDI = \frac{M_w}{M_n}$$

M_w = Weight Average Molecular Weight

M_n = Number Average Molecular Weight

Each sample was divided in two batches and both were subjected to the same characterisation process.

Result are summarised in Table 1.

Tab. 1: Data obtained by GPC measurement

Ratio n(OH groups) : n(monomer)	***hPG_{10k}***	***Mn (kDa)***	***PDI***
1:5	$PPO_{1.54}$-lPG$_{4.09}$	63	1.345
1:5	$PPO_{1.54}$-lPG$_{3.93}$	61	1.301
1:10	$PPO_{1.67}$-lPG$_{3.73}$	60	1.200
1:10	$PPO_{1.67}$-lPG$_{3.83}$	61	1.237
1:20	$PPO_{2.59}$-lPG$_{5.39}$	83.5	1.316
1:20	$PPO_{2.59}$-lPG$_{5.53}$	85	1.259
1:5	$PBO_{1.02}$-lPG$_{1.27}$	32	1.271
1:5	$PBO_{1.02}$-lPG$_{1.32}$	33	1.266
1:10	$PBO_{1.95}$-lPG$_{3.82}$	67	1.340
1:10	$PBO_{1.95}$-lPG$_{3.90}$	67	1.406
1:20	$PBO_{1.25}$-lPG$_{4.30}$	65	1.210
1:20	$PBO_{1.25}$-lPG$_{4.34}$	65	1.213

First of all, it is possible to observe that polymers don't present the expected repeating units.

Moreover, after purification steps, some side products were revealed by the GPC analysis. It is supposed that some starting material was entrapped in the growing polymer and therefore it was not more capable of reacting.

Focusing on the M_n, it is evident the correlation with the chain length, as expected: in fact, M_n grows with increasing number of the repeating units.

Finally, it is important to investigate the polydispersity index. This value represents the width of molecular weight distribution. [49] In general, a PDI of 1 corresponds to a monodisperse polymer, e.g. proteins. Regarding the polymers presented in this work, they all exhibit a PDI between 1.2 and 1.4, which is a desired result as usually polymers obtained through this technique have PDI smaller than 2.[50] Therefore, it is possible to affirm that the synthesis led to a narrow distribution.

3.3 Dye encapsulation

After complete polymer characterisation, tests were made in order to assess the capacity of polymers in loading guest molecules.

Moreover, this experiment permits to compare the role of different architectures. Analyses were conduct using two dyes used as standards,[51] Nile red and pyrene.

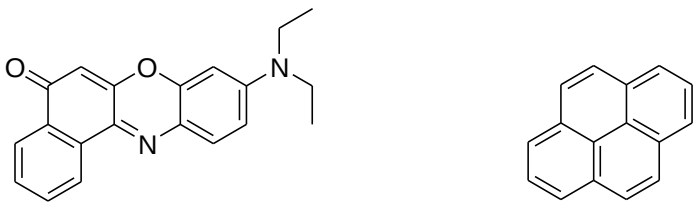

Fig. 17: Representation of Nile red (left) and pyrene (right).

In both cases, the film method was used. A thin layer of dye was obtained through evaporation on vials' wall and an aqueous solution of polymer was stirred there for 24 h. After this period, samples were filtered in order to remove the dye which remained in solution.

This method enables to load both hydrophilic and hydrophobic guest using a restrained amount of organic solvent, which is only necessary for film preparation.[52]

Using UV/Vis spectroscopy it was possible to determinate the quantity of dye contained in the polymers.

At first, encapsulation of Nile red was investigated; however, even if a little amount of dye resulted in entrapped in solution, the concentration of dye encapsulated by the polymer was too low to represent a significant result. It is supposed that, due to the structure of the molecule, there were only little interactions with the CMS and, therefore, it was not able to accommodate a huge amount of guest molecule. Attention was therefore turned to pyrene. Using this dye it was possible to obtain interesting results for the entire library.

The typical UV spectrum for this dye is presented in figure 18.

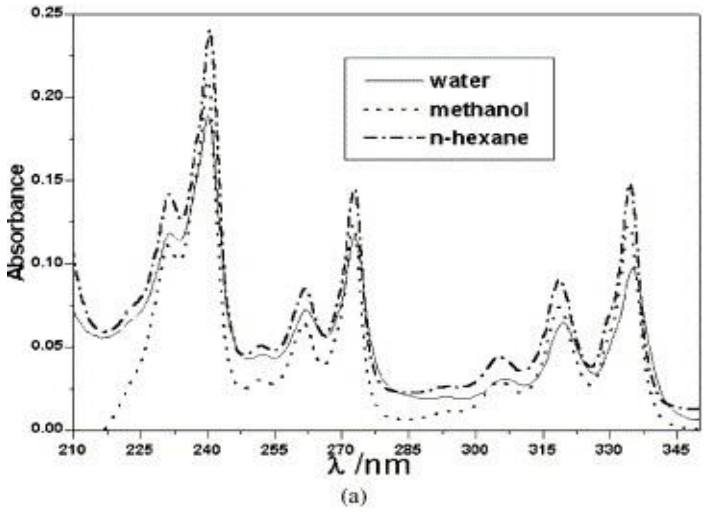

Fig. 18: Example of pyrene spectra in different solvents[53]

Measurements were first conduct in water, to establish if pyrene was present in the samples. Moreover, the behaviour in water is a fundamental parameter as it is the natural medium mostly composing the body and in which the CMS should operate. After this verification, probes were lyophilised and dissolved in methanol. In fact, due to the slight solubility of the dye in water, any extinction coefficient was ever determinate for pyrene in this solvent.
UV/Vis analyses were hence retaken and the concentration of pyrene was determinate.
Finally, the interest was to verify the possibility of a relation between the concentration of polymer and the encapsulation capability. Hence, two solutions of 1 mg/mL and 5 mg/mL were prepared from each polymer.

3.4 Determination of transport capacity

Attention was focused on the transport capacity, meant as amount of guest molecule which can be entrapped and maintained in the polymer architecture. It is expressed as mg of dye per g of polymer.
Concentration of dye in polymers' solution was obtained from the UV/Vis spectra registered in methanol. Values are calculated using the Lambert-Beer equation:

$$A = \varepsilon * C * d$$

A = absorbance
ε = extinction coefficient
c = molar concentration
d = path length in cm

Concentration was determined using the absorbance value relative to the peak at 337nm. The extinction coefficient of pyrene in methanol corresponds to 45.300 $cm^{-1}M^{-1}$;[54][55] the path length is 1 cm. An example of the obtained spectra is presented in figure 19.

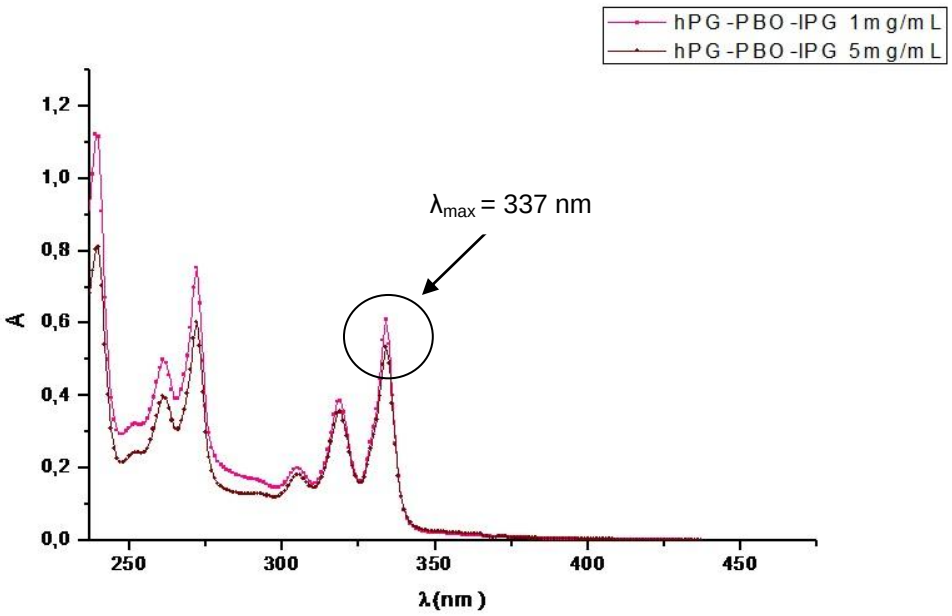

Fig. 19: Pyrene spectra in MeOH, taken at different concentrations.

The spectrum presents the comparison of the performance of a polymer related to the concentration.

In general, all the solution of 5 mg/mL showed an absorbance higher than those of 1 mg/mL; moreover, their peak at 337 nm was out of the linearity range (A>1), so it was necessary to dilute them factor of 6. Measurements were then replicated and spectra were correlated.

Since a little quantity of pyrene could have been dissolved in water instead being contained in the polymers, a “blank” solution was prepared. It corresponds to a solution of pyrene in water, obtained through the film method. Practically, only water was stirred for 24 h and then filtered. The concentration of blank solution was subtracted from the concentration estimated by the graphs.

The following table presents the results.

Tab. 2: Transport capacity of polymers

Polymer	*Mean (mg_{pyr}/g_{pol})*	*Std deviation*
$hPG_{9.9k}$-$PPO_{1.54}$-$lPG_{3.93}$ 1mg/mL	0,665	0,240
$hPG_{9.9k}$-$PPO_{1.54}$-$lPG_{3.93}$ 5 mg/mL	1,062	0,052
$hPG_{9.9k}$-$PPO_{1.67}$-$lPG_{3.83}$ 1 mg/mL	0,919	0,031
$hPG_{9.9k}$-$PPO_{1.67}$-$lPG_{3.83}$ 5 mg/mL	1,512	0,059
$hPG_{9.9k}$-$PPO_{2.59}$-$lPG_{5.53}$ 1 mg/mL	0,398	0,106
$hPG_{9.9k}$-$PPO_{2.59}$-$lPG_{5.53}$ 5 mg/mL	0,869	0,160
$hPG_{9.9k}$-$PBO_{1.02}$-$lPG_{1.32}$ 1mg/mL	2,912	0,331
$hPG_{9.9k}$-$PBO_{1.02}$-$lPG_{1.32}$ 5mg/mL	3,234	0,111
$hPG_{9.9k}$-$PBO_{1.95}$-$lPG_{3.90}$ 1mg/mL	0,484	0,214
$hPG_{9.9k}$-$PBO_{1.95}$-$lPG_{3.90}$ 5mg/mL	1,412	0,213
$hPG_{9.9k}$-$PBO_{1.25}$-$lPG_{4.34}$ 1mg/mL	1,082	0,189
$hPG_{9.9k}$-$PBO_{1.25}$-$lPG_{4.34}$ 5mg/mL	1,303	0,310

It is possible to observe that the highest transport capacity (TC) is exhibited by the polymer $hPG_{9.9k}$-$PBO_{1.02}$-$lPG_{1.32}$, dissolved in a solution of 5 mg/mL.

Moreover, an increase in dye concentration is recognised with the augmentation of polymer in solution.

Due to the interest in the influence of architecture on the transport capacity, these values were related to the DP_n of polymers. However, any correlation between the transport capacity and the chains length was observed.

Attention was therefore pointed at the hydrophilic-lipophilic balance (HLB). HLB represent an empirical calculation based on the groups composing

the molecule; it allows predicting the behaviour of an amphiphilic compound.[56]

According to Griffin, HLB was determined with the following equation:

$$HLB = 20 * \frac{MW_{hydrophob}}{MW_{molecule}}$$

$MW_{hydrophob}$ = molecular weight of the hydrophobic part

$MW_{molecule}$ = molecular weight of the entire molecule

The correlation between the transport capacity and the HLB is displayed in figure 20.

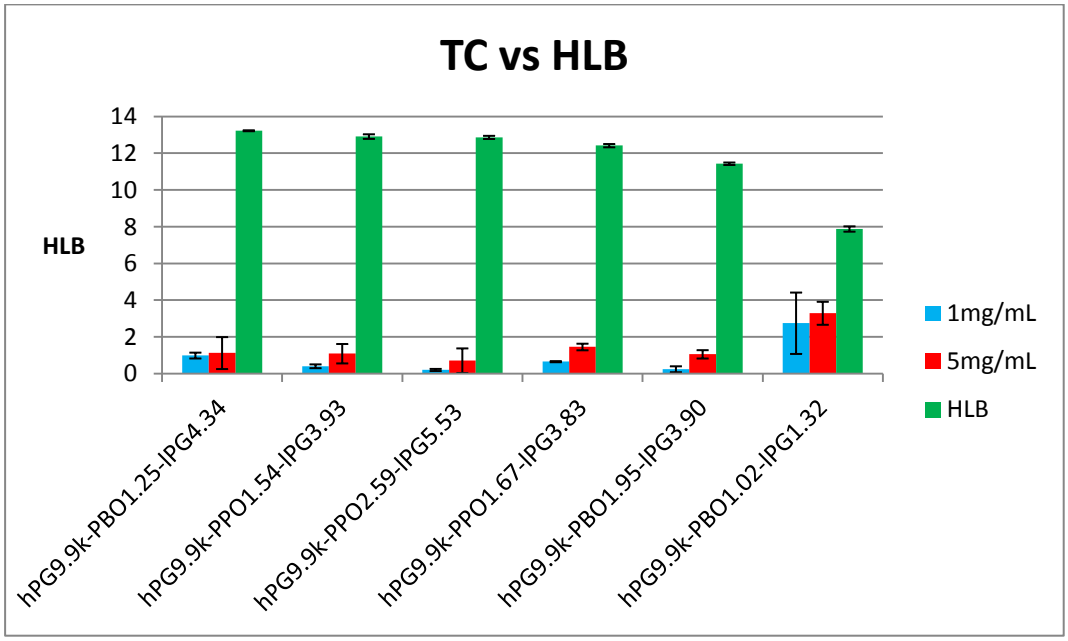

Fig. 20: Diagram relating TC to HLB

The first consideration which derives from the observation of the graphic is that five polymers show a similar value of HLB, around 11 and 13.

The last product presents, on the other hand, a lower value.
Concentrating on the relationship between HLB and TC, it is possible to see that the highest TC is shown by the polymer with the lowest HLB.
This fact can be explained by the affinity that exists between the polymer and the dye. In fact, it is supposed that the highest hydrophobic grade of the polymer leads to a better accommodation of the dye, which is hydrophobic too.

3.5 Encapsulation of Dexamethasone

Due to the interesting behavior shown by the library with regard to pyrene, it was decided to investigate also the encapsulation of Dexamethasone (DXM). This is a corticoid already employed for treating inflammatory and skin disease. The molecule is presented in figure 21.

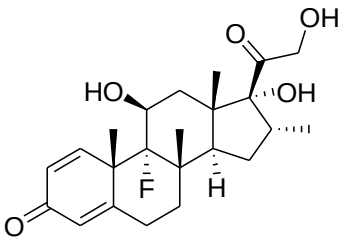

Fig. 21: Structure of DXM

As previously, an aqueous solution of polymer was stirred with the drug and then filtered to remove any excess of DXM. HPLC analyses were conducted at FU Pharmacy Institute, so that the concentration of drug contained in polymers was determined.
The performance of the library was then compared with that of different CMS chosen as benchmark. These samples were obtained in previous

works at FU laboratories. They are CMS made from different starting materials, owning chains of various lengths.
Table 3 presents the obtained data.

Tab. 3: Comparison of different CMS

Batch # CMS polymer	***DXM content (%) in CMS***
$hPG_{9.9k}$-$PBO_{1.95}$-$lPG_{3.90}$	1,05006
$hPG_{9.9k}$-$PBO_{1.02}$-$lPG_{1.32}$	0,76227
$hPG_{9.9k}$-$PBO_{1.25}$-$lPG_{4.34}$	0,68806
$hPG_{9.9k}$-$PPO_{1.67}$-$lPG_{3.83}$	0,53059
$hPG_{9.9k}$-$PPO_{2.59}$-$lPG_{5.53}$	0,52335
$hPG_{9.9k}$-$PPO_{1.54}$-$lPG_{3.93}$	0,40389
DP001-1062	3,45917
DP003-1092f	2,28991
DP003-1098f	2,24828
MU CMS-E-10	2,54874

First of all, it is possible to observe that the polymer $hPG_{9.9k}$-$PBO_{1.95}$-$lPG_{3.90}$ exhibits a better TC than the others.
If a comparison with the encapsulation of pyrene is made, it is evident that the best carrier is not represented by the same polymer.
Nevertheless, the structure of DXM is different from that of pyrene; therefore it appears logic that interactions between the systems differ.
Regarding this behavior, any correlation with HLB was found. On the other hand, depending on the more hydrophilic character of the guest molecule and due to the presence of some hydroxyl groups, it is possible to suppose that in this case also interactions as hydrogen bonds can be formed.
Focusing on the influence of the monomer composing the inner shell, all the products made of PBO result superior in accommodating the drug.

This fact suggests that the choice of the monomer used for constructing the interior can effectively influence the performance of the CMS.
It is evident that there is a huge gap between the performance of the benchmark CMS and the synthesized library. However, considering the possibility of improving the synthesis and reaching a system really showing the desired characteristics, the capability of transporting quite the half amount of the standards appears a promising result.

3.6 Size analysis through DLS measurements

The size of particles was determinate by Dynamic Light Scattering (DLS) technique. Measurements were performed both on the bare samples and after dye loading, in order to compare the results.
Table 4 shows the dimensions of particles determined in aqueous solution.

Tab. 4: Particle size of products

Product	*Before*	*After*
	Size (nm)	Size (nm)
$hPG_{9.9k}$-$PPO_{1.54}$-$lPG_{3.93}$ 1mg/mL	12,19 ± 0,16	11,98 ± 0,26
$hPG_{9.9k}$-$PPO_{1.54}$-$lPG_{3.93}$ 5 mg/mL	10,89 ± 0,22	10,88 ± 0,36
$hPG_{9.9k}$-$PPO_{1.67}$-$lPG_{3.83}$ 1 mg/mL	12,63 ± 0,35	13,68 ± 1,23
$hPG_{9.9k}$-$PPO_{1.67}$-$lPG_{3.83}$ 5 mg/mL	10,51 ± 0,31	10,83 ± 0,09
$hPG_{9.9k}$-$PPO_{2.59}$-$lPG_{5.53}$ 1 mg/mL	15,31 ± 0,66	15,11 ± 0,16
$hPG_{9.9k}$-$PPO_{2.59}$-$lPG_{5.53}$ 5 mg/mL	14,54 ± 0,42	15,55 ± 1,81
$hPG_{9.9k}$-$PBO_{1.02}$-$lPG_{1.32}$ 1mg/mL	9,10 ± 0,72	10,58 ± 0,87
$hPG_{9.9k}$-$PBO_{1.02}$-$lPG_{1.32}$ 5mg/mL	7,91 ± 0,33	9,72 ± 0,52
$hPG_{9.9k}$-$PBO_{1.95}$-$lPG_{3.90}$ 1mg/mL	11,74 ± 0,18	11,62 ± 0,10
$hPG_{9.9k}$-$PBO_{1.95}$-$lPG_{3.90}$ 5mg/mL	9,90 ± 0,34	10,58 ± 0,31
$hPG_{9.9k}$-$PBO_{1.25}$-$lPG_{4.34}$ 1mg/mL	13,99 ± 1,39	14,23 ± 1,37
$hPG_{9.9k}$-$PBO_{1.25}$-$lPG_{4.34}$ 5mg/mL	12,14 ± 0,86	12,47 ± 0,12

The data demonstrate that products present a narrow size distribution, as all the particles are included in the range 8-15 nm.

This is an important fact as size is a fundamental parameter regarding particle absorption. In fact, too small particles will rapidly be excreted by kidneys, while too big compounds will be eliminated by immune system.

Therefore, a good compromise is represented by a size enclosed in the range 10-200 nm.

Moreover, small particles (≤ 30 nm) seem to be able to reach the deepest layer of skin by intercellular route[57].

Considering the importance of size in nanocarriers performance, it is possible to deduce that particles possess the desired dimensions.

Another interesting aspect is represented by determination of size after loading the dye. In fact, none of the polymers exhibits a relevant change in size. Therefore, it is possible to suppose that transport happens by a unimolecular way[58].

This behavior is typical of so called “unimolecular micelles”, which are polymeric structures where surfactants are covalently bound. This permits to obtain micelles-like molecules which are thermodynamically stable[59].

4. Conclusions and outlooks

This work presents a new approach for the synthesis of core-multishell architectures, starting from epoxides. The tedious and time spending process, requiring also an activation step, was substituted with an easier synthetic pathway.

CMS composed of a hPG core, a non-polar inner shell and a hydrophilic outer shell were obtained through anionic ring opening polymerisation.

The “grafting-from” technique was employed to covalently bind the branching on the polyether backbone.

This two-step synthesis results faster and therefore also more economic.

Furthermore, after improvement, the new strategy seems to be suitable for producing CMS in higher quantities as the previous work.

Epoxides were chosen for their high reactivity, due to ring strain. Using propylene oxide and butylene oxide as building blocks it was possible to compare the influence of the monomer on the reaction.

Ethoxyethylglycidyl ether (EEGE) was employed as monomer for constructing the outer shell. It is also suitable for an AROP, so that the previous synthetic path can be maintained. Its polymerisation leads to a hydrophobic product, which can be easily deprotected, obtaining a polar chain.

One of the aims of this work was to produce a library which exhibit increasing chain length of the hydrophobic inner shell. Calculation of the repeating units demonstrates that the goal was only partially reached. However, it is supposed that by improving the reaction, it will be possible to obtain a new synthesis which leads to the target product. This work can be considered a proof of a concept, of the feasibility of a process which needs to be ameliorated. All the polymers were characterised by GPC, NMR and DLS. GPC measurements show that the M_n of the products is related to the repeating units composing them. Furthermore, the

estimation of PDI, resulting in values included between 1.2 and 1.4, which is in the typical range of polymers obtained by ROMB polymerisation.

DLS measurements in water demonstrate that the entire library is composed of small particles in the interval 9-15 nm. Analyses were also repeated after dye loading and any particular change were observed in particles size. This fact convince us that transport happens in a unimolecular way. Moreover, the particles possess dimensions which make them suitable for dermal applications.

In order to compare the performance of the products, the transport capacity of them was tested by encapsulation of dyes.

First of all it was observed that TC is related to the concentration: indeed, a bigger amount of dye was revealed in solution of 5 mg/mL.

Moreover, it was noted that one polymer exhibits a greater capacity in loading dye. In order to determinate its qualities and explain the better behaviour, attention was pointed on the HLB. Correlating the TC with this value it was possible to discover that the polymer possessing the major liphophilic tendency is the best one in encapsulating pyrene. The carrier is made from butylene oxide.

Finally, the performance of the polymers was also tested by encapsulation of a drug for skin diseases, Dexamethasone.

The results demonstrate that the entire library is able to encapsulate it; another time, the best behaviour is shown by the products obtained with butylene oxide. This fact suggests that there is an influence of the monomer in the performance of these architectures.

Moreover, as transport capacity was tested using both an hydrophobic and an hydrophilic guest, it is possible to affirm that the CMS presented in this work exhibit the desired capability of hosting different kind of molecules.

By comparison with the TC of model CMS, it was observed that the polymers require an improvement in order to reach the desired performance. However, the results appear promising.

5. Experimental Part

5.1 Materials and Methods

5.1.1 Reagents

Chemicals

The hyperbranched polyglycerol was obtained by DendroPharm and used as received; the polymer was dissolved in Methanol.

The monomer ethoxyethylglycidyl ether was prepared according to literature,[60] through distillation, using the procedure describe in the following section.

All the other reagents were used as received from the following commercial suppliers: KOtBu pure, 1M in THF (Acros Organics); Propylene oxide, 99.5% extra pure (Acros Organics); Butylene oxide (Sigma Aldrich).

Solvents

All the solvents were obtained by commercial suppliers.

5.1.2 Analytical methods

NMR Spectroscopy

The NMR technique was performed in order to confirm that the expected structures were obtained. Comparisons were made with the spectra from literature. The hyperbranched polyglycerols displayed in the work of Sunder were chosen as model.[61] This allowed affirming that we were able to create the desired products.

Moreover, for those polymers not comparable with literature, affirmations were based on NMR prediction obtained by software.

^{1}H NMR and ^{13}C NMR were recorded at 25°C using an ECP 500 spectrometer (Joel USA, MA, USA). The deutereted solvents used were $CDCl_3$ and DMSO-d6. The chemical shift are given in ppm relative to TMS or the solvent signal (^{1}H NMR: $CDCl_3$: δ = 7.26 ppm, DMSO-d6: 2.50 ppm; ^{13}C NMR: $CDCl_3$ = 77.23 ppm, DMSO-d6 = 39.5 ppm).

GPC

The Gel Permeation Chromatography is a chromatographic technique also known as Size Exclusion Chromatography (SEC).

It is a liquid chromatography in which components are separated according to their size. At first, the biggest molecules are eluted, and then the smaller follow. In fact, if molecules are too big to enter in the pores of the column, they will be rapidly excreted; on the other hand, small molecules will interact with the column and therefore they will exit later.

Usually, a high molecular weight is required to the sample; meanwhile there is no upper limit to its weight[49].

This technique is important because it permits to determinate some characteristics of a polymer, which consents to characterise it: the molar mass and the distribution of molecular weight[49].

Before the analysis, the sample are solubilised in the proper liquid medium and filtered with 0.45 µm PTFE filter.

The GPC consist of a Shimadzu HPLC/GPC machine. Three columns (PPS: Polymer Standards Service GmbH, Germany; Suprema 100A°, 300A°, 1000A° with 5 mm particle size) were used to separate aqueous polymer samples using DMF with 3 gL^{-1} LiBr, 6 gL^{-1} acetic acid as the mobile phase at a flow rate of 1mL min^{-1}. The columns were operated at ambient temperature with the RI detector at 50 °C.

Dinamic Light Scattering

DLS is a technique which enables to measure the size of particles in solution. In fact, measuring the Brownian motions, it is possible to determinate the dimensions of the particle which generate the signal.

The entire process is based on mathematic models.

To measure the particle size it is necessary to use the Einstein – Stokes equation:

$$d(H) = \frac{kT}{3\pi\eta D}$$

$d(H) =$ hydrodynamic diameter

$k =$ Boltzmann's constant

$T =$ absolute temperature

$\eta =$ viscosity

$D =$ translational diffusion coefficient

The motion of the particles in solution causes some fluctuations which indicate the dimension and the distribution of sizes.

To analyze the phenomenon it is necessary to use an autocorrelation function:

$$C(\tau) = B[1 + A * e^{(-2\Gamma\tau)}]$$

$\Gamma =$ line width

$\tau =$ delay time.

The line width Γ can be determined from the slope of autocorrelation function, using the following equations:

$$\Gamma = \mathrm{D} * \mathrm{q};\ q = \frac{4\pi n}{\lambda_0} \sin\left(\frac{\theta}{2}\right)$$

$q =$ scattering wave vector

$\lambda_0 =$ laser's wavelength

$\theta =$ measuring angle of scattered light.

As the scattering wave vector is a constant, due to set up of measurements, it is possible to acquire the diffusion constant.
The DLS analyses were performed using a Zetasizer Nano instrument (Malvern Instrument, United Kingdom).
The chosen laser wavelength was λ = 632 nm and the temperature was kept constant at 25°C.
After a first measurement, samples were filtered in order to remove possible interferences due to dust particles and the measures repeated.

UV-VIS

UV-VIS measurements were performed in order to investigate the transport capacity of the carriers. In fact, starting from the absorbance values, it is possible to calculate the concentration of dye loaded in the product.
The UV/vis spectra were recorded on a PerkinElmer LAMBDA 950 UV/vis/NIR spectrometer.
The carriers were at first tested in water solution, to confirm the load of the dye; the probes were then lyophilised and dissolved in methanol, in order to conduct a quantitative measurement.
The Lambert-Beer's Law was employed to determinate the effective concentration of dye loaded in the carrier.

$$A = \varepsilon \cdot c \cdot d$$

A = absorbance
ε = extinction coefficient
c = molar concentration
d = pathlenght in cm

As pyrene is a little soluble in water, a referring solution was prepared, so that it is possible to determinate the amount of dye which can be

solubilised. All the measurements were correct by subtracting this value from the obtained results.

HPLC

The Quantitative determination of Dexamethasone in CMS was obtained by HPLC analyses, using the following conditions.

A Column Lichrochart RP 18 (Fa. Merck), with particle size of 5µm and pore size of 100 A was used.

An eluent composed of Acetonitrile and Water in ratio 40% to 60% was chosen. The flow rate was of 0.5 mL/min and a volume of 20µL was injected. The detector was set at 254nm.

In order to obtain a quantitative determination, external standards of Dexamethasone solved in Acetonitrile (100µg/ml, 50µg/ml, 25µg/ml, 10µg/ml, 5 µg/ml and 0.5µg/ml) were prepared.

5.2 Synthesis

Hyperbranched PG was prepared in our laboratory through a ROMB synthesis.[18]

5.2.1 Synthesis of inner shell

The same recipe was used both for the preparation of polypropylene oxide and polybutylene oxide.

The synthesis was performed according to literature.[61]

Briefly, under inert condition, hPG-OH was heated to 60°C, in presence of KOtBu; as the reagents were dry, temperature was increased to 95°C and N-methyl-2-pyrrolidinon was added. The epoxide was added drop wise over 4h. The solvent was evaporated under reduced pressure.

Tab. 5: Ratio between initiator and monomer amount

	n(OH groups) [mmol]	n(PO) [mmol]		n(OH groups) [mmol]	n(BO) [mmol]
Product			**Product**		
hPG-PPO 1:5	13,5	68	**hPG-PBO 1:5**	13,5	68
hPG-PPO 1:10	13,5	136	**hPG-PBO 1:10**	13,5	136
hPG-PPO 1:20	13,5	272	**hPG-PBO 1:20**	13,5	272

Spectrum DP008-0001d

hPG-PPO: ^{1}H NMR (500 MHz, DMSO-D6) δ 4.39-4.3 OH, 3.7-3.16 CH,CH_2, 1.03, -0.99 CH_3. ^{13}C NMR (126 MHz, DMSO-D6) δ 74.85-72.40 CH, CH_2, 65.53-65.39 CH_2, 48.79, 48.76, 20.34-17.36 CH_3.

Spectrum DP008-0012d

hPG-PBO: ^{1}H NMR (500 MHz, CHLOROFORM-D) δ 4.5 OH, 3.59-3.27 CH,CH_2, 1.46-1.22 CH_2 PBO, 0.92, - 0.89 CH_3. ^{13}C NMR (126 MHz, CHLOROFORM-D) δ 74.00-71.47 CH, CH_2, 50.61, 29.76- 24.62 CH_2, 9.90 CH_3.

5.2.2 Synthesis of outer shell

The synthesis was performed according to literature[60]. 2.3-epoxypropanol (72.6 g, 980 mmol) was dissolved in ethylvinyl ether (365.5 ml, 3801 mmol) and TsOH (1.71 g, 9 mmol) was added. The product was recovered by liquid-liquid extraction with a saturated solution of $NaHCO_3$. The organic solution was dried with Magnesium sulphate then evaporated under reduced pressure. The product was distilled under vacuum, yielding 109.49 g (yield: 76.53 %) of a transparent liquid.

Spectrum DP008-0003
^{1}H NMR (500 MHz, CHLOROFORM-D) δ 4.70-4.65 CH, 3.75-3.40 CH_2 (t), 3.39-3.31 CH_2, 3.06-2.71 CH ring, 2.57-2.52 CH ring, 1.25-1.10 CH_3. ^{13}C NMR (126 MHz, CHLOROFORM-D) δ 99.37-99.35 CH, 65.48-60.32 CH_2, 50.58-50.47 CH ring, 44.24, 44.18, 19.65-14.94 CH_3.

5.2.3 Grafting from poly(EEGE) to the inner shell

Briefly, under inert condition, hPG-PPO was heated to 60° C, in presence of KOtBu; as the reagents were dry, temperature was increased to 95°C and N-methyl-2-pyrrolidinon was added. The epoxide was added drop wise over 4h. The solvent was evaporated under reduced pressure.
The same path way was applied for the synthesis starting from hPG-PBO.

Spectrum DP008-0005def
hPG-PPO-lPG: ^{1}H NMR (700 MHz, DMSO) δ 4.60-4.2 OH, 3.7-3.35 backbone, 1.05 CH_3. ^{13}C NMR (176 MHz, DMSO) δ 80.04-74.51 CH, 72.44-60.84 backbone, 17.17 CH_3.

Spectrum DP008-0015edh
hPG-PBO-lPG: ^{1}H NMR (500 MHz, DMSO-D6) δ 4.64-4.3 OH, 3.75-3.25 backbone + solvent, 1.50-1.38 CH_2 PBO, 0.86 CH_3. ^{13}C NMR (126 MHz, DMSO-D6) δ 80.17 CH, 71.76-60.98 backbone, 24.24 branching, 9.48 CH_3.

Tab. 6: Ratio between inner shell and monomer amount

	n(hPG-PPO) [mmol]	n(EEGE) [mmol]		n(hPG-PBO) [mmol]	n(EEGE) [mmol]
Product			**Product**		
hPG-PPO-lPG 1:5	68	68	**hPG-PBO-lPG 1:5**	68	68
hPG-PPO-lPG 1:10	68	68	**hPG-PBO-lPG 1:10**	68	68
hPG-PPO-lPG 1:20	68	68	**hPG-PBO-lPG 1:20**	68	68

5.3 Deprotection

In order to obtain the final product, deprotection of the outer shell was performed. The protecting group is represented by an etoxyethylether, which is labile in acid medium[62].

First, the polymers are dissolved in an organic solvent. Depending on the polarity's grade of the product, the chosen solvents are methanol and dichloromethane. pH one is reached adding trifluoroacetic acid. The solution is kept stirring for 48 h, afterwards the solvent is evaporated under reduced pressure.

5.4 Purification

The entire products have been purified using the dialysis technique.

This is a physical chemical technique which exploits the concentration gradient to separate molecules dissolved in a liquid medium.

The dialysis employs a semi-permeable membrane to extract the molecules which are under a certain molecular weight and therefore can be considered undesired products.

It was chosen to use dialysis tubes with different cut off: 3.5 kDa, 4-6 kDa.

In general, the sample is first dissolved in a proper solvent, and then transferred in a dialysis tube. The tube is placed in a beaker filled with the corresponding solvent and dialysis is performed at least for 24 h.
The hPG-PPO 1:1 was dissolved and dialysed in MeOH; hPG-PPO 1:2 was dissolved in MeOH but dialysed in a solution 1:1 of MeOH-DCM. All the others products were both dissolved and dialysed in a solution 1:1 of MeOH and DCM.
To ensure a better dialysis process, at first we tried to perform it in a basic ambient, provided adding triethylamine and reaching pH 8. In fact, due to the different ambient in the tube and surrounding it, the expulsion of side product should be favour.
Since GPC analysis revealed that some products were not completely purified another dialysis was performed after the deprotection step. In this case it was chosen to use a saturated solution of NaCl in water, as it was supposed that the saline solution would help in breaking the aggregates; during the two days of dialysis the concentration of salt was gradually halved until using only water. For this process, dialysis tubes with cut off 14 kDa were used.
However, through dialysis, it was not possible to completely purify the final products; in particular, polymers which possess both the shells still present some undesired products even after repeated dialysis.
It was therefore supposed that harder conditions were required to expel entirely the side product, so it was decide to perform ultra filtration.

5.5 Ultrafiltration

This process enable to separate large molecules suspend in a liquid medium from smaller ones. It exploits the pressure to eliminate the undesired particles through a semi-permeable membrane.
The procedure used a membrane with a cut off of 10 kDa.

Polymer is dissolved in a solution 50:50 of water and methanol. Once the sample is placed in the cell, pressure is applied through an inert gas. Solution is so forced to pass through the membrane and ejected from the cell. Solution is filled up two times with the initial solution, then thirteen times with only water. Once the process is completed, the remaining solution is recovered and solvent evaporated under reduced pressure.

5.6 Encapsulation of dyes

In order to test the loading capability of the product, encapsulation of the dye Nile red was performed. The process was operated by the film method. This represents a general method of encapsulation which can be used for all the dye.

First, a solution of dye in methanol is prepared. A certain amount of solution is then transferred in vials and the solvent is evaporated in an oven at 75°C for two hours. Via this process, a tiny stripe of dye remains on the glasses.

The second step consists of the solubilisation of a desired amount of product in different solvents (water and PBS); 3 mL of solution are transferred in each vial and stirred for 24 h, at 1200 rpm.

After this time, the products are recovered, filtered with a RC filter of 0.2 µm and analysed by UV-spectroscopy.[5]

The carriers were also tested using pyrene; the same encapsulation step way was performed.

First, a solution of dye in dimethyl ether is prepared. The solution is then transferred in some vials and the solvent is evaporated. The second step consists of the solubilisation of a desired amount of product in different water; 3 ml of solution are then transferred in each vial and stirred for 24 h. After that period, solutions are filtered with RC filter of 0.2 µm.

As pyrene is slightly water soluble, a blank is prepared transferring 3 mL of water in one of the vials, without the polymer.

5.7 Encapsulation of DXM

Transport capacity was also tested using a drug, Dexametasone.
A solution of DXM in Ethanol (c = 15 g/L) was prepared, then was put in vials containing the polymers. In particular, 1/5 DXM compared to the mass of the CMS was added.
Solutions were treated with ultrasound for different time periods, in order to homogenise them.
The ethanol was removed in a drying oven at 40 °C over 15 hours, themìn MQ water was add.
The samples were stirred for 24h at 1200 rpm, and then were filtered through 0.45 um RC syringe filters in order to remove any excess of DXM.
The samples were transferred to Stefan Hönzke and Anja Elpelt FU Pharmacy for HPLC analysis of the DXM content.

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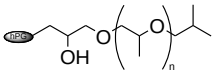
7. Appendix

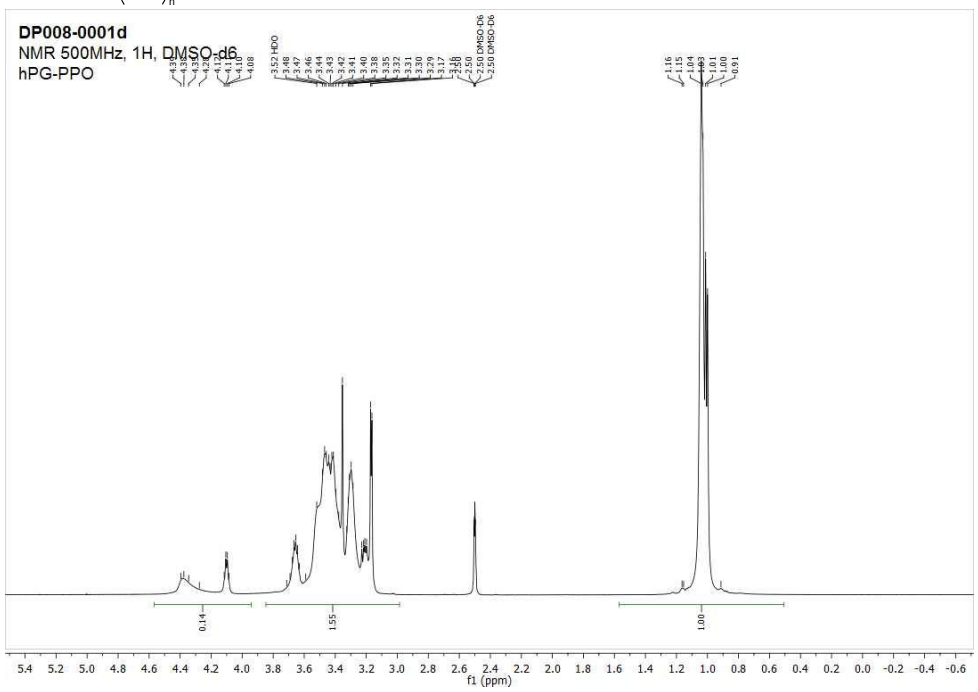
7.1 Abbreviations

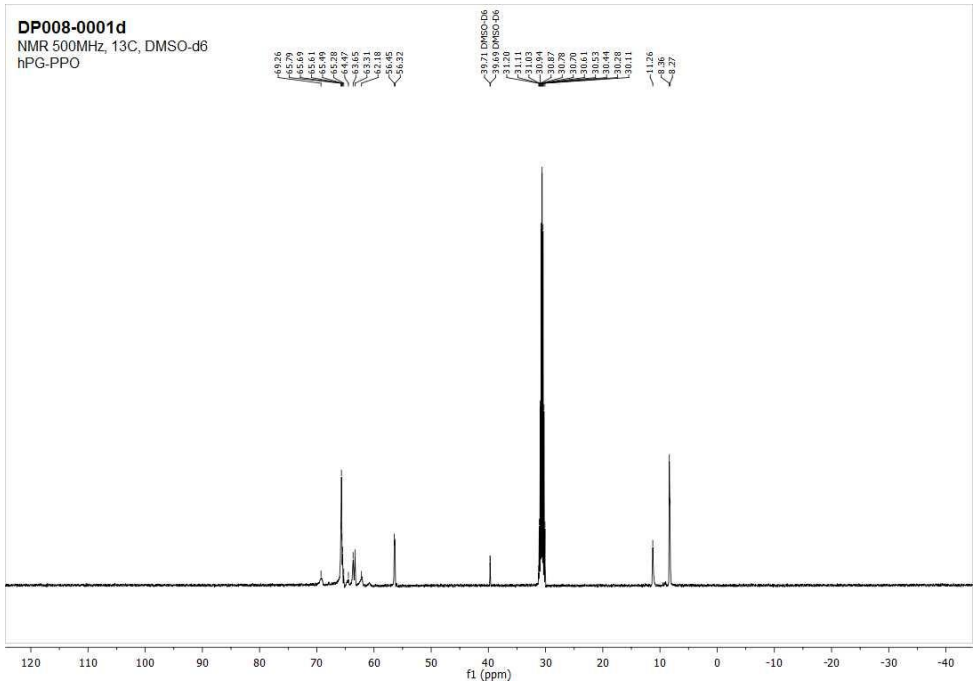
PEG	Poly(ethylene glycol)
FDA	Food and Drug Administartion
hPG	Hyprebranched polyglycerol
CMS	Core-multishell architectures
EEGE	ethoxyethylglycidyl ether
CMC	Critical Micelle Concentration
CAC	Critical Aggregation Concentration
EPR	Enhanced permeability and retention
ROP	Ring-opening polymerization
AROP	Anionic ring-opening polymerization
PTSA	p-Toluenesulfonic acid
PEI	Poly(ethylene imine)
mPEG	monomethyl Poly(ethylene glycol)
NMP	N-Methyl-2-pyrrolidone
DP_n	Degree of polymerisation
GPC	Gel Permeation Cromatography
Mn	Number Average Molecular Weight
Mw	Weight Average Molecular Weight
PDI	Polydispersity index
MeOH	Methanol
TC	Transport capacity
HLB	Hydrophilic-lipophibic balance
DXM	Dexamethasone
HPLC	High performance liquid cromatography
FU	Freie Universität
PBO	Poly(butylenes oxide)

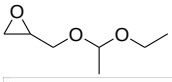
DLS	Dinamic light scattering
PPO	Poly(propylene oxide)
NMR	Nuclear magnetic resonance
KOtBu	Potassium tert-butoxide
THF	Tetrahydrofuran
ROMB	Ring-opening multibranching
SEC	Size Exclusion Chromatography
PTFE	Polytetrafluoroethylene
PG	Polyglycerol
PO	Propylene oxide
BO	Butylene oxide
TsOH	p-Toluenesulfonic acid
$NaHCO_3$	Sodium bicarbonate
DCM	Dichloromethane
NaCl	Sodium chloride
PBS	Phosphate-buffered saline
rpm	Repetitions per minute
RC	Regenerated cellulose

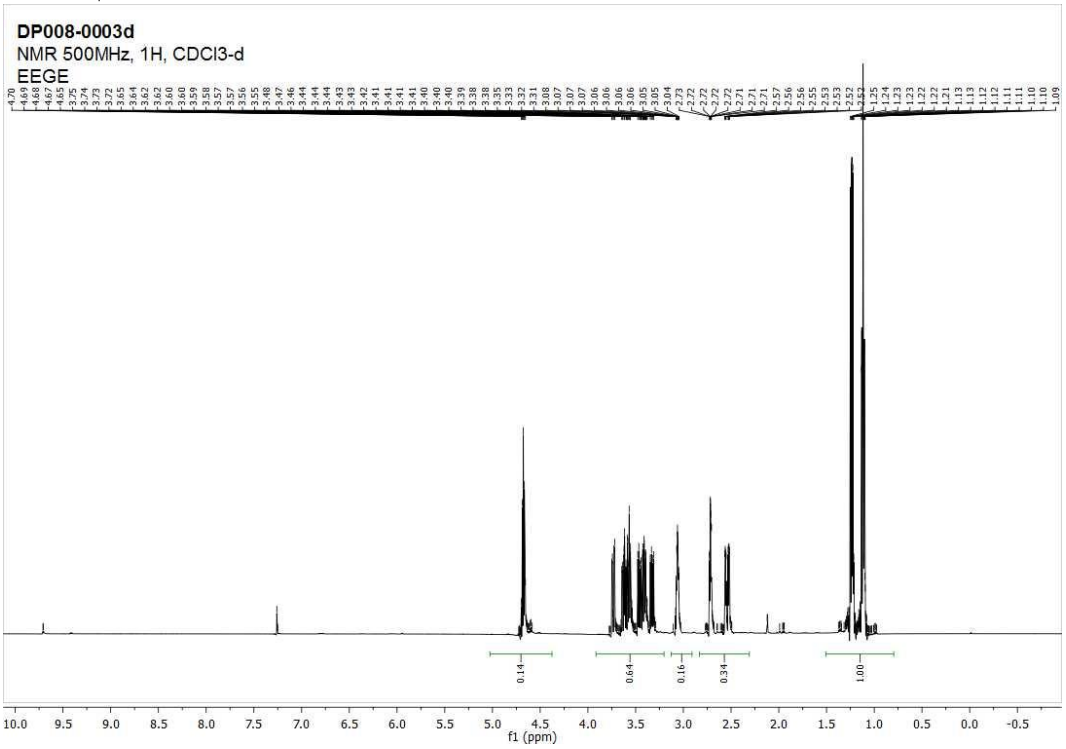
7.2 NMR spectra







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EEGE
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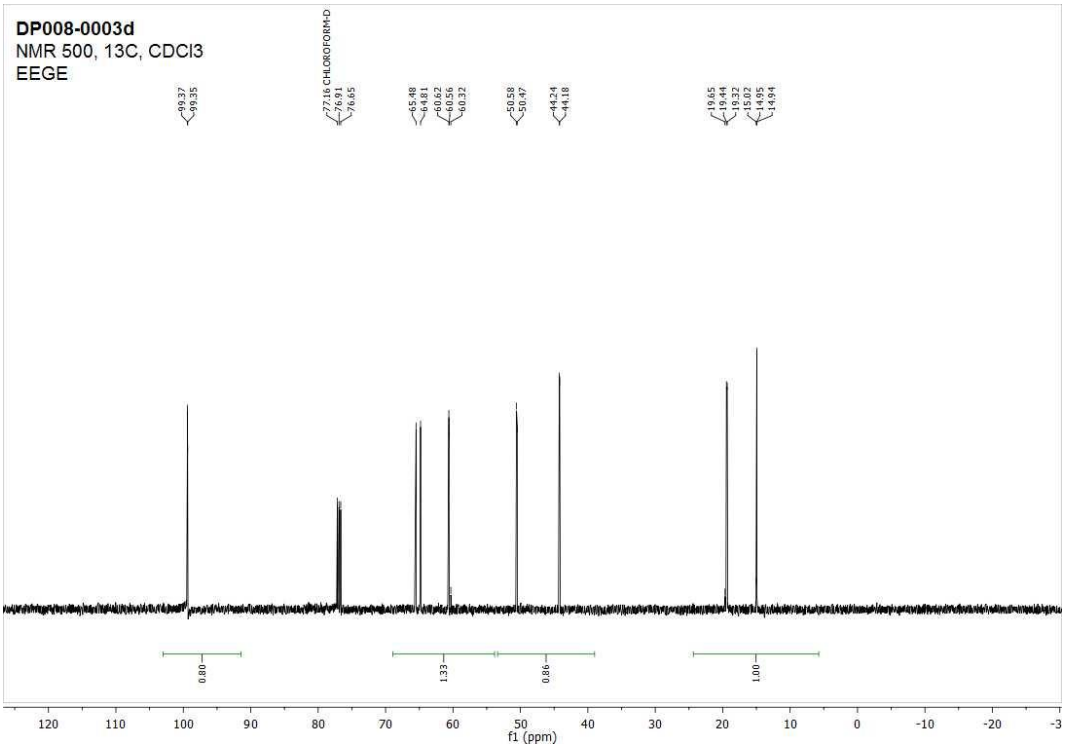
0.16
0.34
1.00
f1 (ppm)


DP008-0003d
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EEGE
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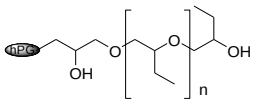
1.33
0.86
1.00
f1 (ppm)

Appendix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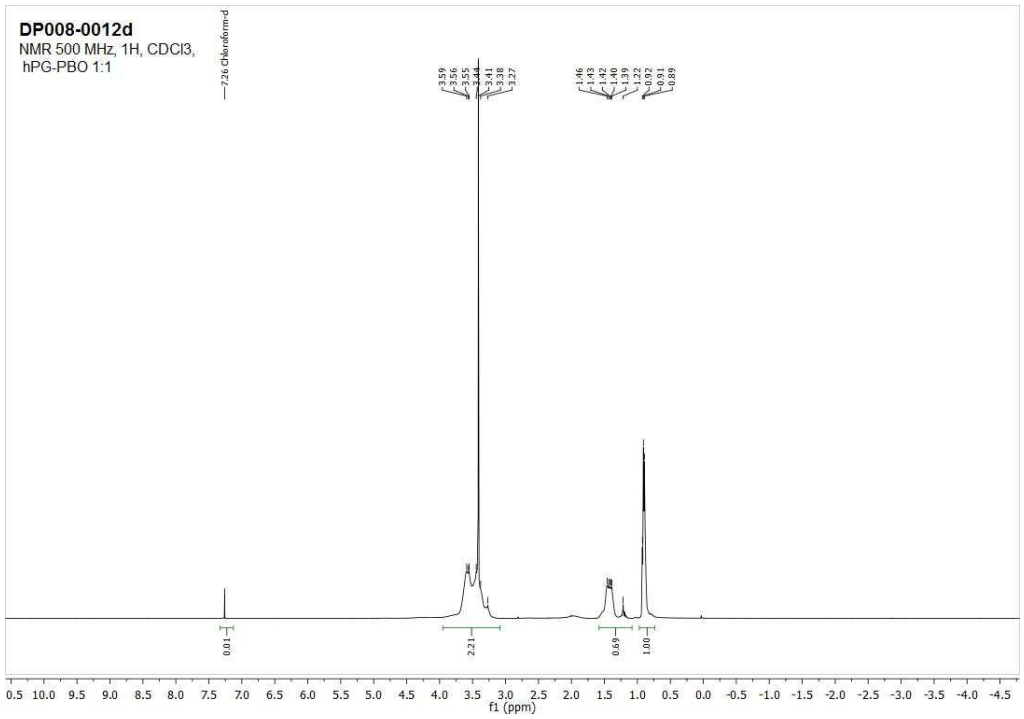
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3.56
3.55
3.44
3.41
3.38
3.27
1.46
1.43
1.42
1.40
1.39
1.22
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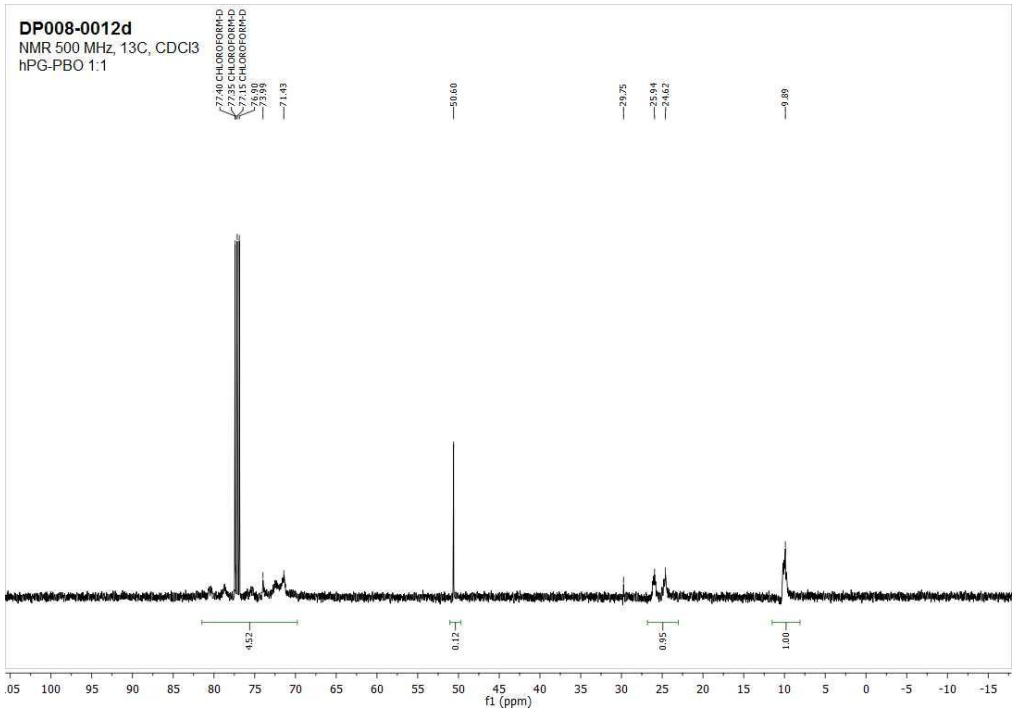
0.91
0.89
0.01
2.21
0.69
1.00
f1 (ppm)


DP008-0012d
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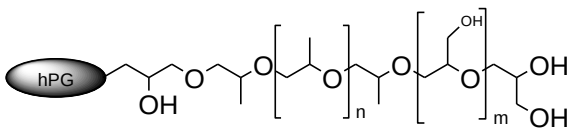
hPG-PBO 1:1
77.40 CHLOROFORM-D
77.35 CHLOROFORM-D
77.15 CHLOROFORM-D
73.99
71.43
50.60
29.75
25.94
24.62
9.89
4.52
0.12
0.95
1.00
f1 (ppm)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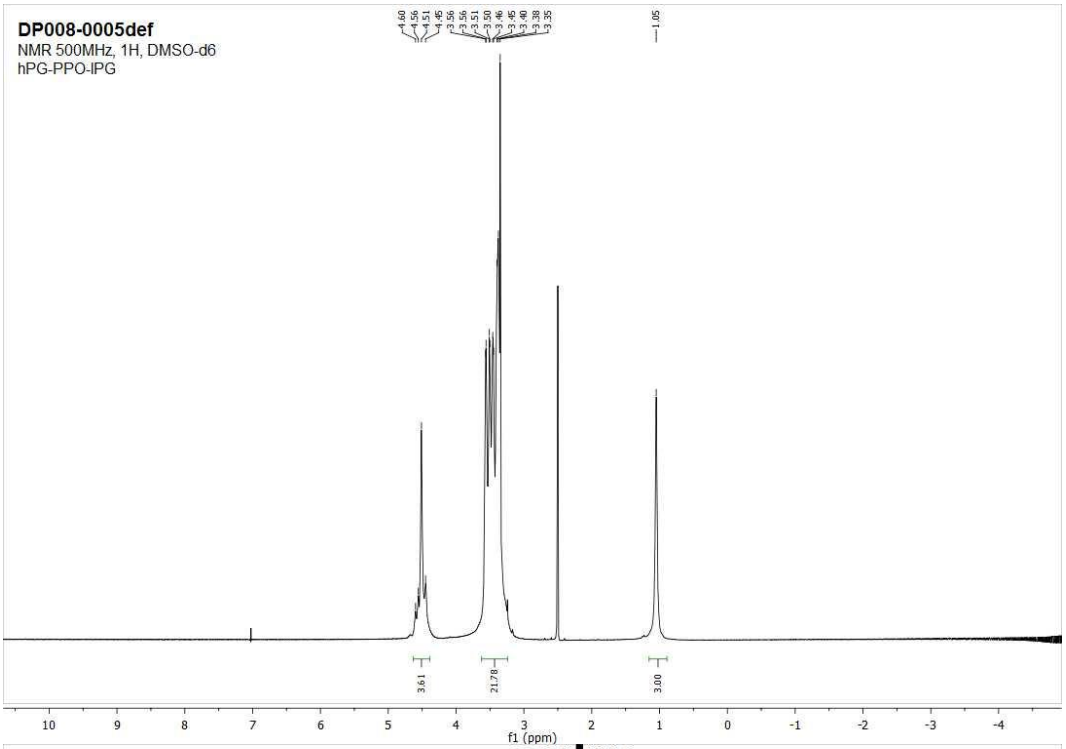
OH
OH
n
m
OH


DP008-0005def
NMR 500MHz, 1H, DMSO-d6
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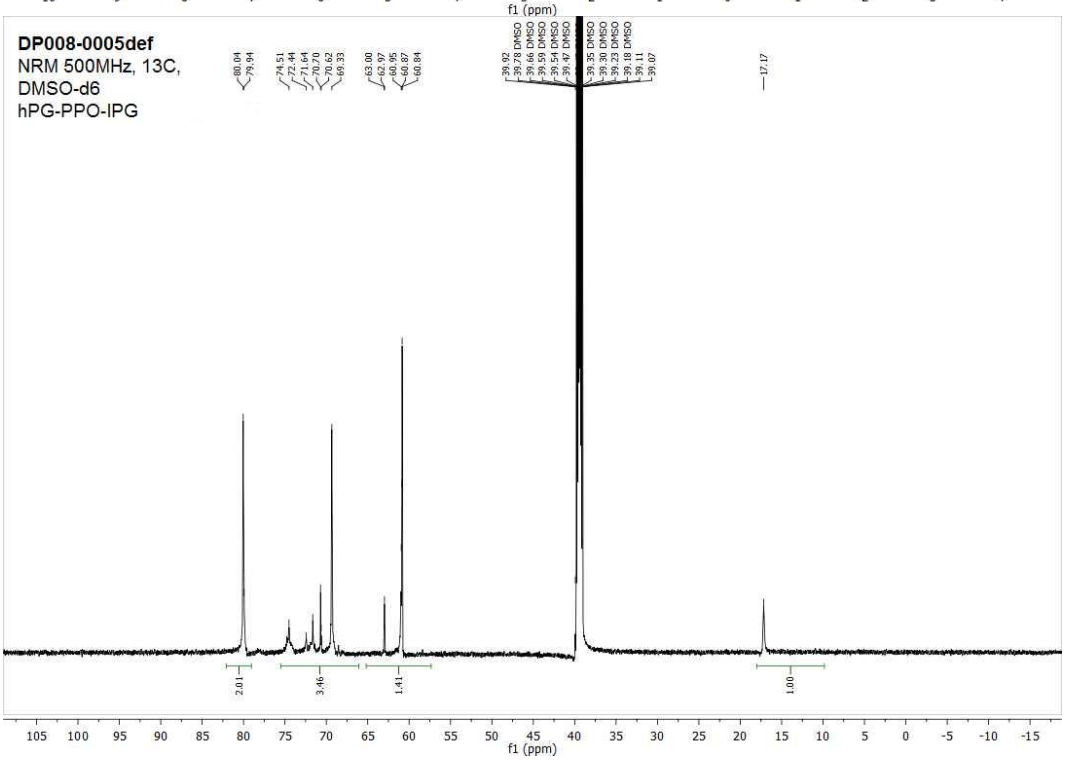
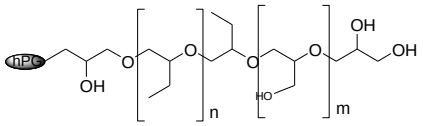
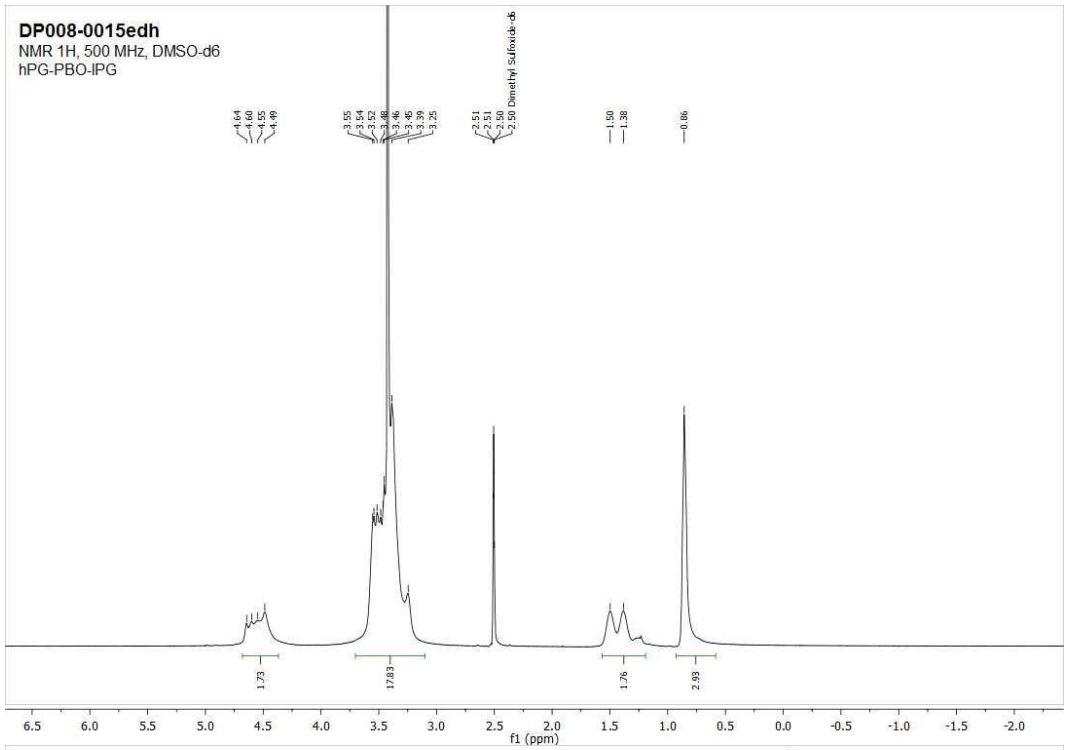
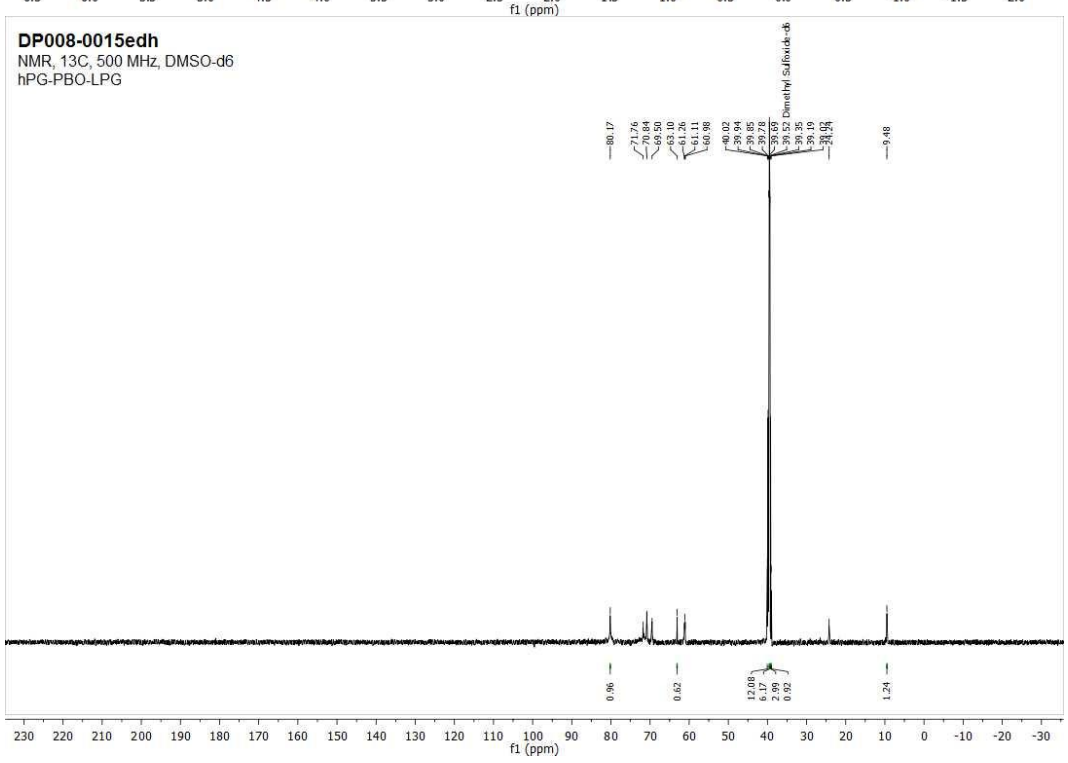
4.56
4.51
4.45
3.56
3.51
3.50
3.46
3.45
3.40
3.38
3.35
1.05
3.61
21.78
3.00
f1 (ppm)

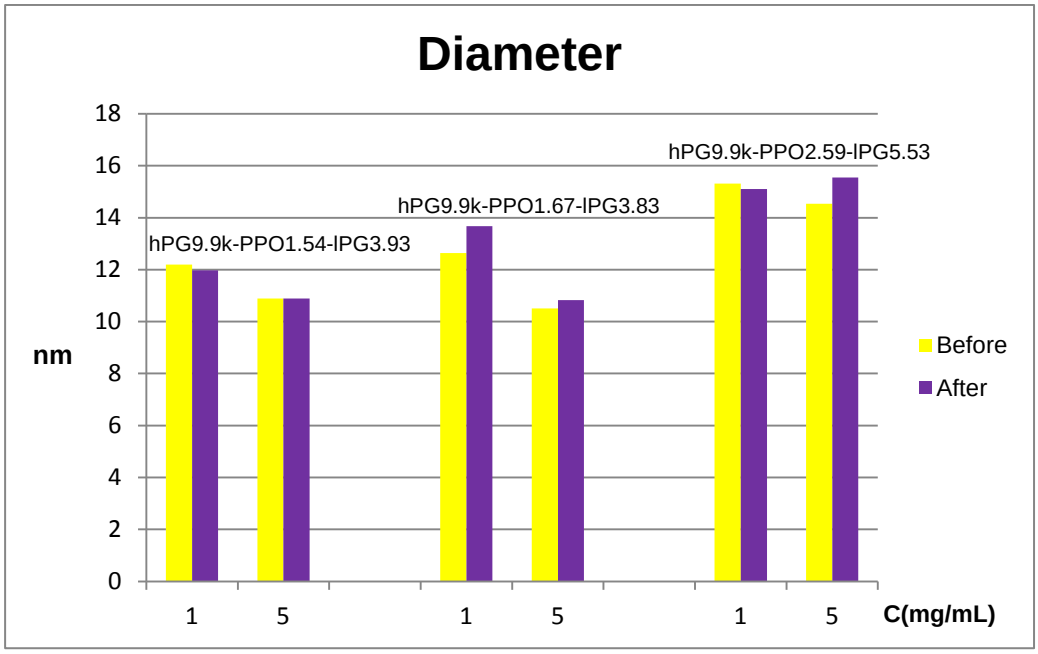

DP008-0005def
NRM 500MHz, 13C,
DMSO-d6
hPG-PPO-lPG
80.04
79.94
74.51
72.44
71.64
70.70
70.62
69.33
63.00
62.97
60.95
60.87
60.84
39.92
39.78
39.64
39.59
39.54
39.47
39.35
39.30
39.23
39.18
39.11
39.07
17.17
2.01
3.46
1.41
1.00
f1 (ppm)

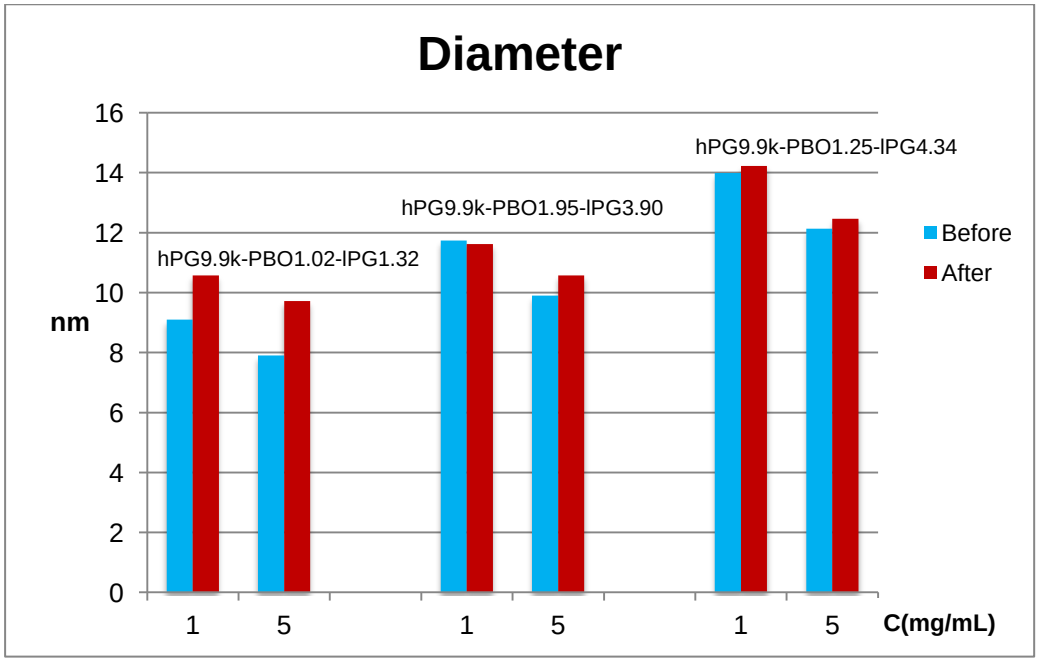

hPG
OH
O
O
O
O
OH
OH
HO
n
m


DP008-0015edh
NMR 1H, 500 MHz, DMSO-d6
hPG-PBO-lPG
Dimethyl Sulfoxide-d6
1.73
17.83
1.76
2.93
f1 (ppm)


DP008-0015edh
NMR, 13C, 500 MHz, DMSO-d6
hPG-PBO-LPG
Dimethyl Sulfoxide-d6
0.96
0.62
12.08
6.17
2.99
0.92
1.24
f1 (ppm)

7.3 DLS


Diameter
18
16
14
12
10
8
6
4
2
0
nm
hPG9.9k-PPO1.54-lPG3.93
hPG9.9k-PPO1.67-lPG3.83
hPG9.9k-PPO2.59-lPG5.53
1
5
1
5
1
5
C(mg/mL)
Before
After


Diameter
16
14
12
10
8
6
4
2
0
nm
hPG9.9k-PBO1.02-lPG1.32
hPG9.9k-PBO1.95-lPG3.90
hPG9.9k-PBO1.25-lPG4.34
1
5
1
5
1
5
C(mg/mL)
Before
After