SYNTHESIS AND CHARACTERIZATION OF POLYMERIC BIODEGRADABLE MICELLES FOR TARGETED DRUG DELIVERY

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“Do not kick away the canoe which helped you cross the river” (Madagascar)

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LIST OF ABBREVIATIONS

A: absorbance
ABCPA : 4,4-azobis-(4-cyanopentanoic acid)
ACN: acetonitril (CH$_3$CN)
AmAc: ammonium acetate
°C: degree Celcius
c.a. : circa
CDCl$_3$: deuterochloroform (deuterated chloroform)
CLSM: Confocal Laser Scanning Microscopy
CMC: critical micelle concentration
CP: Cloud Point
DCC : N,N'-dicyclohexyl carbodiimide
DCM: dichloromethane
DLS: Dynamic Light Scattering
DMF: dimethylformamide
DPTS : 4-(dimethylamino)pyridinium-4-toluene sulfonate
DTT: dithiothreitol
d: extinction coefficient
EDTA: ethylenediaminetetraacetic acid
e.g.: exempli gratia
EGa1: nanobody against EGFR
EGFR: Epidermal Growth Factor
EPR: Enhanced Permeability and Retention
etc.: et cetera
eq.: equivalent
Fabs: fragments of antibodies
GPC: Gel Permeation Chromatography
HPLC: High Performance Liquid Chromatography
HPMAmDL: N-(2-hydroxyethyl)methacrylamide dilactate
i.d.: internal diameter
LiCl: lithium chloride
mAbs: monoclonal antibodies
$M_n$: number average molar weight
\[ M_p \]: peak average molecular weight
MPS: Mononuclear Phagocyte System
\[ M_w \]: weight average molar weight
NMR: Nuclear Magnetic Resonance
PBS: Phosphate Buffered Saline
PDI: Polydispersity Index
PDP: pyridyldithiopropionate
PEG: poly (ethylene glycol)
ppm: parts per million
RhoMA: rhodamine methacrylate
RI: Refractive Index
RT: Room Temperature
scFv: single chain variable fragments
SATA: N-succinimidyl-S-acetylthioacetate
SDS: sodium dodecyl sulphate
SDS-PAGE: sodium dodecyl sulphate - poly acrylamide gel electrophoresis
SPDP : N-succinimidyl-3-(2-pyridyldithio)-propionic acid
TAIC: trichloroacetyl isocyanate
THF : tetrahydrofurane
TMS: tetramethylsilane
1. INTRODUCTION

1.1. GENERAL INTRODUCTION

Up to now the three most common treatments of cancer are surgical intervention, chemotherapy and radiation. The principal aim of the last two is to stop the proliferation and migration of malignant cells. Chemotherapy in particular, has limitations, because it affects healthy rapid dividing cells too, which results in severe adverse effects. These circumstances have led to the development of tumour-targeted delivery systems for intravenous administration of anticancer drugs, to improve their therapeutic efficacy.

Ideally, these drug carriers have to accumulate exclusively in the diseased tissue and offer a controlled release of the drug in an adequate concentration. This controlled release can make it possible to achieve the same therapeutic response with a smaller dose. At the same time there is a decrease of the undesirable side effects and an increase of the bioavailability in the pathological area. To reach a therapeutic effect, these drug delivery systems should have a high drug loading capacity, and should be stable in the blood stream for a prolonged period of time. Another desirable property of the vehicle is a minimal toxicity and an increased biodegradability, after the pharmaceutical agents have been released.

1.2. POLYMERIC MICELLES AS DRUG DELIVERY SYSTEMS

After pioneering work during the last quarter of the twentieth century, the ability of polymeric micelles to act as an efficient drug delivery system has been scientifically investigated. Micelles have first been studied as drug carriers by the group of Kataoka in 1992. They have gained increased attention as vehicles, because in some cases they are more suitable than other delivery systems, as described in paragraph 1.2.3.
1.2.1. Passive targeting

The enhanced permeability and retention (EPR) effect is a passive targeting ability. It is the property by which micelles accumulate in tumour tissue but not in healthy tissues. The EPR effect was described by Maeda and Matsumura in 1986. The general explanation for this phenomenon is based on two factors. Firstly, because tumour cells grow quickly, they induce angiogenesis in the pathological tissue, because they secrete various growth factors. These newly formed blood vessels have a higher permeability due to their discontinuous endothelium, which forms wide fenestrae. The size of fenestrations in tumour capillaries can be up to 700 nm (Moghimi et al., 2001).

Secondly, tumour tissues additionally have an insufficient lymphatic drainage, which means that there is less backward flow into the blood capillaries (Maeda, 1992). These two factors lead to fluent transport of micelles through the hyperpermeable endothelial layer into the pathological area and subsequent retention there (figure 1.1.), because the micelles have a size larger than the cut-off value of the fenestrae of normal blood vessels and smaller than the ones of tumour vessels (Fisher et al., 2007).

![Diagram of drug delivery](image)

1.2.2. Prolonged circulation time

It is important to notice that to achieve passive targeting and depot properties, micelles have to circulate in the bloodstream for a prolonged period of time, so that a sufficient amount of the particles can penetrate into the tumour. Two important parameters for the prolonged circulation time are: the micellar size and the steric stabilisation.

Concerning the size, particles with a diameter less than 5 to 10 nm are eliminated by renal excretion. On the other hand, nanospheres larger than 200 nm are in particular distributed to the spleen by mechanical filtration (Kabanov et al., 2002). So, polymeric micelles with a diameter between 10 and 100 nm can have prolonged circulation times.

Steric stabilisation involves the coating of the particle surface with hydrophilic polymers such as PEG. Metselaar et al. (2003) studied the effect of surface functionalization of colloidal drug carriers with PEG chains on the biological fate of the particles. They found that grafting the surface with PEG prolonged the in vivo blood circulation half-life, because it reduced the non-specific removal by the complement system and by the mononuclear phagocyte system (MPS). Since the two main driving forces for opsonisation are electrostatic and hydrophobic interactions, the effect of PEGylation is probably due to the steric and hydrophilic shielding of the surface charge. It is not yet clear which mechanisms are responsible for this phenomenon. It is important to notice that the smaller the micelles, the higher the surface density of PEG and hence the longer the circulation time.

1.2.3. The advantages of polymeric micelles as drug delivery systems

In the field of drug delivery and targeting, the recently progressive interest in the use of micelles has five reasons. Firstly, the hydrophobic core has a high loading capacity for hydrophobic drugs. Secondly, their small size permits them to be injected intravenously in order to reach target tissues through leaky capillaries, which leads to an accumulation in the pathological areas. Thirdly, the presence of the hydrophilic
brush on the surface and also the small size prolongs the circulation time. Fourthly, after releasing the therapeutic molecules, polymeric micelles dissociate in the body into single block copolymer chains. The molecular weight of the resulting polymer, lower than the threshold for excretion, guarantees the removal by renal clearance to prevent their long-term accumulation in the body (Seymour et al., 1987 and Lavasanifar et al., 2002). Fifthly, a specific ligand can be coupled on the outer surface of the micelles, to achieve active targeting.

1.2.4. Characteristics of the used polymers and micelles

In this study, thermosensitive amphiphilic diblock copolymers were synthesized, consisting of a hydrophilic and a thermosensitive segment, PEG and HPMAmDL respectively. An aqueous solution of thermosensitive polymers is characterized by a so-called cloud point (CP) and by a critical micelle concentration (CMC). The CMC is the concentration whereby the self-assembly of micelles takes place. The CP is the temperature below which the thermosensitive block is hydrated, and hence soluble in water. Once above the CP of the thermosensitive part, the hydrogen bonds between water and the thermosensitive block are disrupted making it hydrophobic, which results in the formation of micelles with a PEG shield and a HPMAmDL core. The advantage of the use of these thermosensitive block copolymers is that, they can form micelles by simply heating an ice-cold aqueous polymer solution (at a concentration above the CMC) to a temperature above the CP, for one minute. This technique is called the rapid heating method (Neradovic et al., 2004). Hence, no (toxic) organic solvents are required.

It is important to have possibilities to control the CP. The CP of the polymer can be modulated by copolymerizing with hydrophobic or hydrophilic molecules. Hydrophobic moieties decrease the CP, whereas hydrophilic moieties have an opposite effect (Shibayama et al., 1996). pHPPAmDL-b-PEG in particular has a CP between 7 - 10 °C, far below the body temperature. It is expected that block copolymers of HPMAmDL and PEG form polymeric micelles at 37°C (Soga et al., 2004). HPMAmDL is a well-known, non-toxic macromolecule which has already been used for the development of drug carriers of cytostatic agents (paclitaxel). These
systems demonstrated promising results in clinical trials at the beginning of the twenty-first century (Vasey et al., 1999; Duncan, 2003).

It is possible to control the instability of micelles composed of pHPMAmDL-\(b\)-PEG at body temperature, because due to the hydrolysis of the hydrolytically sensitive ester bond of HPMAmDL, the CP rises above the body temperature whereby the polymers become water soluble and the micelles start dissolving (Neradovic et al., 2001). For these characteristics and for all the reasons mentioned before, these amphiphilic polymers are attractive materials for drug carriers, because they degrade into non-toxic and water soluble products. A schematic representation of polymeric micelles with controlled instability is shown in figure 1.2.

![Figure 1.2: Schematic Representation of Polymeric Micelles with Controlled Instability](image)

1.3. COUPLING OF NANOBODIES ON THE MICELLES FOR ACTIVE TARGETING

Although the long circulation time promotes the passive targeting of the micelles and the delivery of the entrapped drug, the accumulation of the pharmaceutical agents at the pathological area is rather low. Rijcken et al. found in 2007 that the accumulation, due to the EPR effect, the small size and the prevention of opsonisation thanks to the PEG coating, in an in vivo study was only 6% of
the injected dose in mice, 48 hours after intravenous administration. Hence, active targeting to promote the site specific action would be interesting, to obtain new micellar systems that are able to distinguish healthy and malignant cells.

Parallel to the research on targeted drug delivery systems, further investigations have been done in the field of tumour molecular biology. Epstein et al. (1995) discovered that the structure of the vasculature in solid tumours is not the only abnormality. Carcinomas are associated with more surface antigens than healthy tissue. For instance, there is an over-expression of the epidermal growth factor receptor (EGFR), a transmembrane glycoprotein with an intracellular tyrosine kinase domain (Yasui and Imai, 2008). Tyrosine kinases are enzymes that phosphorylate the amino acid tyrosine, and play an important role in the regulation of cell proliferation, angiogenesis, etc. Hence, these receptors are interesting in the field of anticancer therapy, which have led to EGFR-targeted therapeutics to inhibit the EGFR activation (Traxler, 2003). Several monoclonal antibodies, which interfere with ligand binding to a receptor, have been screened for their ability to bind to the EGFR (Modjtahedi and Dean, 1994).

Ideally, the ligand binding to the antigen stimulates the endocytic uptake of the whole carrier, and once inside the cell the chemotherapeutic agents are released in a controlled manner. Previously, monoclonal antibodies (mAbs, figure 1.3.), fragments of antibodies (Fabs, consist of C\textsubscript{H}1, V\textsubscript{H}, C\textsubscript{L} and V\textsubscript{L}), or single chain variable fragments (scFv, consist of V\textsubscript{H} and V\textsubscript{L}) have been coupled to liposomes and have shown promising results in preclinical studies (Sofou and Sgourous, 2008).

In this study, nanobodies (figure 1.3.) against the over-expressed EGFR were used. Nanobodies are isolated from the heavy-chains of camelid antibodies which can be easily grown in bacteria or yeast. Heavy-chain antibodies contain a single variable domain (V\textsubscript{HH}) and two constant domains (C\textsubscript{H}2 and C\textsubscript{H}3). The cloned and isolated V\textsubscript{HH} domain is termed nanobody. This new engineering of nanobodies was performed in 1993 by Hamers-Casterman et al. These peptides have four advantages compared with regular antibodies: they have a smaller size, are chemically more stable, are more water soluable than Fab or scFv, and are more resistant to heat, pH, proteases, etc. Despite their small size, they possess the full
antigen-binding specificity and affinity towards their receptor as a mAbs (De Genst et al., 2006). Oliviera et al. (2008) demonstrated a removal of 90% of the EGFR at the cell surface of tumours after an incubation time of four hours with liposomes coupled to nanobodies.

Due to their small size, the nanobodies (MW of 15 kDa) show an efficient tissue penetration (Huang et al., 2008). On the other hand, because 15 kDa is below the threshold for glomerular filtration, it leads to relatively fast in vivo blood clearance via the kidneys. However, nanobodies can be easily joined to each other to increase their molecular weight and to prolong their half-life in the bloodstream for several weeks. Roovers et al. (2007) linked up to four nanobodies to create multivalent assemblies.

Here, the used nanobody is EGa1, an antagonist of EGFR (Hofman et al., 2008). In order to be able to synthesize polymer-protein conjugates, the polymer has to be functionalized at first, so that groups on the micellar shell are present for the covalent linkage with a protein (van Dijk et al., 1999). Researchers have developed a variety of techniques for attaching ligands to liposome surfaces (Forssen et al., 1998). Analogous techniques can be used for covalent coupling of ligands to
micelles. In this study, the linking reagent SPDP was used, whereby thiol groups were introduced on HO-PEG$_{5000}$-NH$_2$ in a derivatization step. After the introduction of functional groups on HO-PEG$_{5000}$-NH$_2$, the molecule is esterified with ABCPA, resulting in the macrorinitiator (PDP-PEG)$_2$-ABCPA. This macrorinitiator induces the radical polymerization of HPMAmDL, resulting in the thermosensitive biodegradable block copolymer pHMAmDL-$b$-PEG-PDP.

The SPDP method was first described by Carlsson et al. (1978). This pyridyl disulphide derivate has an amine-reactive portion, namely the N-hydroxysuccinimide ester, with which any molecule with a primary amine, such as HO-PEG$_{5000}$-NH$_2$ can be modified. Thiol containing peptides can be linked via the reactive PDP moieties. Thiol groups are highly appealing, because they react almost exclusively and quantitatively with thiol under physiological conditions (Dufresne et al., 2005).
2. OBJECTIVES

Since most common treatments of cancer are not target specific and cause severe adverse effects, there is a growing interest in the development of micelles as tumour-targeted nanoscopic delivery systems of anticancer drugs. This has led to the main aim of this study: the synthesis and characterization of polymeric biodegradable micelles for drug delivery, coupled with specific targeting ligands for their use in active drug targeting.

In order to be able to couple a ligand to the micelles, at first the polymer has to be functionalized. Thiol groups were introduced to HO-PEG$_{5000}$-NH$_2$ by means of a pyridyl disulphide derivative, namely SPDP. In the subsequent treatment with DTT, the moles of PDP moieties per moles PEG could be determined, based on spectrophotometry, because DTT cleaves the disulphide bond in the PEG-PDP molecule and a pyridine-2-thione molecule is split off. This compound has a molar absorptivity of 8,080 at 343 nm (Stuchbury et al., 1975). The amount of released pyridine-2-thione, is equivalent to the contents of 2-pyridyl disulphide groups attached to HO-PEG$_{5000}$-NH$_2$.

The synthesis of the macroinitiator, (PDP-PEG)$_2$-ABCPA, was achieved by DCC-coupling. In this DPTS-catalysed esterification reaction, HO-PEG$_{5000}$-PDP was grafted to ABCPA. The molecular weight of the biPEGylated ABCPA and its distribution were determined by NMR and GPC.

The next step was the radical polymerization in order to obtain the thermosensitive amphiphilic block copolymers pHMAmDL-b-PEG-PDP. NMR and GPC were used for the further characterization of the polymer.

The determination of the cloud point of an aqueous solution of the polymer was carried out on a UV spectrophotometer coupled to a temperature controller. The absorbance was measured every 0.2 °C during heating up to a temperature of 15°C.

The thermosensitivity of the HPMAmDL block, makes it possible to prepare micelles by the simple rapid heating procedure, which only requires the heating of an
ice-cold aqueous polymer solution (in concentration above the CMC) to 50°C, for one minute. The size of the micelles and their size distribution (polydispersity index, PDI) were determined by dynamic light scattering (DLS).

Finally, the nanobodies EGa1 against the EGFR, which is over-expressed on tumour tissue surface, were coupled to the micelles. Since only thiol containing molecules can be linked to the reactive PDP moieties, present on the termini of the block copolymers, EGa1 was first modified in order to introduce protected sulphydryl groups, by means of reaction with SATA. Afterwards, these sulphydryl groups were deprotected, generating a thioacetylated peptide. Finally, the nanobody-micelle conjugation reaction was performed, via a disulfide bond formation. During these conjugation reactions, the molecule pyridine-2-thione is cleaved. So once again, through the molar absorptivity of the cleaved molecule, the moles protein per moles PDP-polymer could be determined.
3. MATERIALS AND METHODS

3.1. MATERIALS

N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), dithiothreitol (DTT), 4,4-azobis(4-cyanopentanoic acid) (ABCPA), lithium chloride (LiCl) and deuterochloroform (CDCl$_3$) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Acetonitrile (ACN), tetrahydrofurane (THF, purified by distillation before use), dichloromethane (DCM, dried on molecular sieves before use) and dimethylformamide (DMF) were obtained from Biosolve (Valkenswaard, The Netherlands). N,N’-dicyclohexylcarbodiimide (DCC), trichloroacetyl isocyanate (TAIC) and ethylenediaminetetraacetic acid (EDTA) were products of Acros (Zwijndrecht, The Netherlands). Phosphate buffered saline (PBS, contained 0.1 M phosphate, pH 7.5, with 0.1 M NaCl) was purchased from Braun (Melsungen, Germany). The modified poly(ethylene glycol) (HO-PEG$_{5000}$-NH$_2$) was obtained from NOF Corporation (Kyoto, Japan). Ammonium acetate (AmAc) and the molecular sieves (0.4 nm) were products of Merck (Haarlem, The Netherlands). The 0.45 μm filters were purchased from Schleider and Schuell MicroScience GmbH (Dassel, Germany). Rhodamine-methacrylate was obtained from PolySciences Europe (Eppelheim, Germany). N-succinimidyl-S-acetylthioacetate (SATA) was a product from Pierce Biotechnology (Rockford, USA). 4-(dimethylamino) pyridinium-4-toluene sulfonate (DPTS) was prepared as reported by Moore et al. (1990). N-(2-hydroxyethyl) Methacrylamide monodilactate (HPMAmDL) was synthesized as described previously by Neradovic et al. (2003).

For the removal of volatile solvents, a rotavapor from Heidolph Instruments (Schwabach, Germany) was used. Gel filtration was carried out on a Sephadex™ G-25 PD-10 column with a cut-off of 5000 kDa and a sample volume of 2.5 ml (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The thiolated PEG, the macroinitiator and the polymers were collected after freeze drying with a Christ Alpha 1-2 freeze dryer (Salm and Kipp, Breukelen, The Netherlands). A Shimadzu UV-245 spectrophotometer (Hertogenbosch, The Netherlands) was used to measure the UV-absorbance of chemical compounds. $^1$H NMR spectra were recorded on a Gemini 300 MHz spectrometer (Varian Associates Inc. NMR Instruments, Palo Alto, CA). The
centrifuge was a Sigma Laboratory Centrifuge 4K15 (Salm and Kipp, Breukelen, The Netherlands). Gel permeation chromatography (GPC) was carried out on a Waters 2695 Alliance (Waters Associates Inc., Milford, MA) with a PLgel 5 \( \mu \)m polystyrene packed MIXED-D column characterized by 5 \( \mu \)m particle size, 300 mm x 8 mm i.d. (Polymer Laboratories, Middelburg, The Netherlands). The PEG standards were also products from Polymer Laboratories. The GPC was associated with a Waters 2414 Refractive Index Detector or a UV detector (Waters Associates Inc., Milford, MA) and Empower Software Version 1154. The dynamic light scattering method was carried out on a Malvern ALV CGS-3 with a He-Ne laser source (Malvern Instruments, Malvern, U.K.).

3.2 METHODS

3.2.1. Derivatization of HO-PEG\textsubscript{5000}-NH\textsubscript{2}

3.2.1.1. Thiolation of HO-PEG\textsubscript{5000}-NH\textsubscript{2} by means of SPDP

The hydrophilic segment of the amphiphilic micelle-forming copolymers is poly (ethylene glycol), a synthetic polymer with a molecular weight of 5,000 g/mol. In this project, the bifunctional HO-PEG\textsubscript{5000}-NH\textsubscript{2} was used as a starting compound.

In order to be able to synthesize polymer-protein conjugates, the polymer has to be functionalized so that end groups are present for the covalent linkage with a protein (van Dijk et al., 1999). Thiol groups were introduced in a derivatization step with SPDP. The SPDP method was first described by Carlsson et al. in 1978. This pyridyl disulphide derivative has an amine-reactive portion, namely N-hydroxysuccinimide ester, so that any molecule with a primary amine, such as HO-PEG\textsubscript{5000}-NH\textsubscript{2}, can be modified (figure 3.1).

Two molar equivalents of SPDP were added to a solution of HO-PEG\textsubscript{5000}-NH\textsubscript{2} at a concentration of 50 mg/ml in ACN. After stirring overnight (18 hours) at room temperature, the solvent was evaporated using a rotavapor. The product after evaporation consisted of HO-PEG\textsubscript{5000}-PDP but also of unreacted HO-PEG\textsubscript{5000}-NH\textsubscript{2}.
and SPDP. The latter was removed by gel filtration (PD-10 column, cut-off 5,000 kDa, sample volume 2.5 ml), after dissolving the product in water. Afterwards, the product was collected with freeze drying.

![Chemical structure]

**FIGURE 3.1: THIOLATION OF HO-PEG<sub>5000</sub>-NH<sub>2</sub> BY MEANS OF SPDP.**

### 3.2.1.2. DTT assay

The amount of 2-pyridyl disulfide attached to the PEG termini was determined using the reducing reagent DTT. 10 µl DTT (15 mg/ml in PBS) was added to a solution of HO-PEG-PDP (0.5 mg/ml in PBS). PBS was used, because the DTT cleavage of the disulfide bond can be performed most efficiently at pH 7-9 (Carlsson et al., 1978). By this reduction with DTT, a pyridine-2-thione molecule is split off (figure 3.2). This compound has a molar absorptivity of 8,080 at 343 nm (Stuchbury et al., 1975), what makes it possible to determine its concentration by means of UV spectrophotometry. The amount of released pyridine-2-thione is equivalent to the contents of 2-pyridyl disulphide groups attached to PEG.

![Chemical structure]

**FIGURE 3.2: THE DISULFIDE BOND OF HO-PEG-PDP CAN BE CLEAVED BY THE REDUCING REAGENT DTT. THE CLEAVED PYRIDINE-2-THIONE GROUP HAS A MOLAR ABSORPTIVITY OF 8,080 AT 343 NM.**

The UV absorbance at 343 nm of the HO-PEG-PDP sample was measured compared to the blank (PBS), before and after the addition of DTT, using a Shimadzu
UV-245 spectrophotometer. By calculating the change in absorbance ($\Delta A$), the molar ratio of SPDP versus PEG was calculated using equation (1).

When the absolute concentration of the PDP groups ($M$) was needed (paragraph 3.2.5.), the calculations were based on equation (2).

\[
\frac{\Delta A \cdot M_w \cdot HO - PEG - PDP}{\varepsilon \cdot l \cdot [HO - PEG - PDP]} = \text{moles of SPDP per mole of HO - PEG - NH}_2
\]

\[
c = \frac{\Delta A}{\varepsilon \cdot l}
\]

where:
- $\Delta A$: change in absorbance at 343 nm, before and after addition of DTT
- $\varepsilon$: the extinction coefficient of pyridine-2-thione at 343 nm: 8,080 M$^{-1}$cm$^{-1}$
- $M_w$ PEG-PDP: molecular weight of HO-PEG-PDP (mg/mmmole)
- [PEG-PDP]: concentration of HO-PEG-PDP in PBS (mg/ml)
- $c$: concentration of PDP-groups (M)
- $l$: path length of the cuvette (1 cm)

### 3.2.2. Synthesis of macroinitiator

#### 3.2.2.1. The DCC coupling method

The synthesis of the macroinitiator (PDP-PEG)$_2$-ABCPA was achieved using DCC-coupling. In this DPTS-catalysed esterification reaction HO-PEG$_{5000}$-PDP was grafted to ABCPA, after the formation of an active ester. The overall reaction is shown in figure 3.3.

One molar equivalent ABCPA and three molar equivalents of DCC were dissolved in a mixture of 1:1 dry THF and DCM, together with 0.5 molar equivalents of DPTS. After 20 minutes stirring at room temperature (during which the active ester is formed, see figure 3.4), two molar equivalents of PDP-functionalized PEG were added (approximately 500 mg). The desired final total concentration was 100 mg/ml. This mixture was stirred for 24 hours at room temperature and under nitrogen atmosphere, to prevent the presence of water from the air.
The coupling reagent DCC reacts with carboxylic acids like ABCPA, and forms the activated ester (figure 3.4). The formed ester is activated, because substitution with any nucleophile, such as the free hydroxyl group in HO-PEG-PDP (figure 3.5), expels the very stable urea as leaving group. Urea precipitates, because it is not soluble in THF/DCM (Clayden et al., 2001).
FIGURE 3.5: REACTION OF HO-PEG-PDP WITH THE ACTIVE ESTER, RESULTING IN THE FORMATION OF THE MACROINITIATOR.

In this synthesis, the removal of water is very important because, the hydroxyl group of water can also react with the active ester giving PDP-PEG-ABCPA as side product instead of bisubstituted ABCPA. To prevent this, THF was freshly distilled, DCM was dried over molecular sieves (0.4 nm), and the used glassware and molar sieves were dried in the oven.

After purification by filtration over a paper filter to remove the precipitated urea, the solvent was removed under reduced pressure. The remaining solid after evaporation was dissolved in water (stirring for two hours at room temperature) and filtered over hyflo. In this way the unreacted DCC, ABCPA and DPTS were removed. In the end, the product contains (PDP-PEG)$_2$-ABCPA, some unreacted HO-PEG-PDP, but also some monosubstituted ABCPA, which was formed by traces of water attacking the active ester.

The macroinitiator was recovered by freeze drying the filtrate. The yield of this reaction was estimated after weighing the dry product. NMR and GPC were used for further characterization of the macroinitiator.
3.2.2.2. $^1$H NMR

The $^1$H NMR spectra were recorded using a Gemini 300 MHz spectrometer. At first, a $^1$H NMR spectrum was made of the macroinitiator dissolved in CDCl$_3$ (approximately 10 mg/ml). The peak positions were given in parts per million (ppm), relative to tetramethylsilane as an internal standard.

From Rijcken et al. (2007), the chemical shifts ($\delta$, in ppm) in $^1$H NMR spectrum of the macroinitiator are already known: $\delta_{\text{PEG}}$ 3-4 (448 protons of PEG), $\delta_{\text{ABCPA}}$ 1.63 + 1.7 + 2.2-2.6 (14 protons of ABCPA). Consequently, it is possible to estimate from this spectrum the molar ratio of ABCPA per PEG, using equation (3).

$$\text{ratio PEG : ABCPA} = \frac{\text{integral PEG}}{\text{no. } ^1\text{H PEG}} \cdot \frac{\text{integral ABCPA}}{\text{no. } ^1\text{H ABCPA}}$$

(3)

where: integral PEG: area under the peaks (via integration) ascribed to the PEG protons, which refers to the number of protons
no. $^1$H PEG: number of protons in the PEG molecule (448)
integral ABCPA: the area under the curve at $\delta$ 1.63 + 1.7 + 2.2-2.6 in the $^1$H NMR spectrum
no. $^1$H ABCPA: number of protons of ABCPA (14)

To determine the amount of unreacted HO-PEG-PDP, a second $^1$H NMR spectrum was recorded after the addition of some droplets of TAIC to the NMR sample tube. The in situ derivatization of the free hydroxyl groups with TAIC is a simple procedure for the quantitative determination of the unreacted HO-PEG (HO-PEG-PDP or the starting compound HO-PEG-NH$_2$). This method has been used before by, for instance, Loccufier et al. (1991), Ronda et al. (1994), Postma et al. (2006) and Rijcken et al. (2007). The esterification of the hydroxyl compounds produces a downfield shift of the nuclear resonance peaks of the methylene protons alpha to the hydroxyl group (figure 3.6). TAIC, being aprotic causes no additional signals in the spectrum.
FIGURE 3.6: DERIVATIZATION OF THE HYDROXYL END GROUPS OF UNREACTED HO-PEG-PDP WITH TAIC.

3.2.2.3. Gel permeation chromatography

The molecular weights and their distribution of (PDP-PEG)$_2$-ABCPA were determined by GPC, carried out on a Waters 2695 Alliance, with a PLgel 5 µm polystyrene packed MIXED-D column, characterized by 5 µm particle size, 300 mm x 8 mm i.d. The mobile phase was DMF containing 10 mM LiCl. The samples were dissolved at a concentration of 5 mg/ml in the eluent and filtered through a 0.45 µm filter. Other measurement conditions were: isocratic flow rate 0.7 ml/min, column temperature 40°C, and injection volume 50 µl. The retention time was measured by the Waters 2414 Refractive Index Detector, and the peak areas were determined with Empower Software Version 1154.

Prior to the GPC chromatography of the macroinitiator, a calibration curve was obtained with a set of well-characterized PEG standards, whose molecular weights ranged from 100 to about one million g/mole. The calibration curve was obtained by plotting the logarithms of the known molecular masses of the standards against their elution times.
3.2.3. Block copolymer synthesis

3.2.3.1. Radical polymerization

The pHPMAmDL-b-PEG block copolymers were synthesized by radical polymerization (figure 3.7) using HPMAmDL as monomer and (PDP-PEG)$_2$-ABCPA as radical initiator, according to the procedure as previously described for the synthesis of related block copolymers (Neradovic *et al.*, 2001).

HPMAmDL and (PDP-PEG)$_2$-ABCPA were dissolved at a total concentration of 300 mg/ml in acetonitrile. The ratio of monomer to initiator was 150:1 mol/mol to obtain block copolymers with the desired pHPMAmDL block length.

FIGURE 3.7: SYNTHESIS OF pHPMAmDL-b-PEG-PDP BLOCK COPOlyMER BY RADICAL POLYMERIZATION.
The mixture was stirred in an oil bath of 70°C for 20 hours in airtight glass vials and under nitrogen atmosphere. The nitrogen atmosphere and the airtight glass is necessary, because by heating oxygen forms radicals too, which inhibit the polymerization. This should result in an earlier stop of the reaction, which is only controlled by amounts of the reagents.

After the reaction, the mixture was added dropwise to diethylether. The polymers together with unreacted HO-PEG-PDP and (PDP-PEG)$_2$-ABCPA precipitate, while the unreacted monomers dissolve in the ether. The residue was collected by centrifugation, using a Sigma Laboratory Centrifuge 4K15. The supernatants, containing unreacted monomers, were transferred into a weighed round bottom flask. After removal of the solvent by rotary evaporation, the amount of unreacted monomers could be estimated by weighing the flask again. The residue containing the polymers was dissolved in water and purified by dialysis (M$_w$ cut-off 12,000-14,000 Daltons) against deionized water at 4°C, a temperature lower than the cloud point, to prevent the formation of micelles. The water was refreshed every four hours at least 3 times in total. Afterwards, the solution was freeze dried in order to collect the dry product.

The polymers were characterized by $^1$H NMR and GPC. The cloud point was determined using the Shimadzu UV-245 spectrophotometer. To check if the SPDP moieties were still present, the DTT assay was used again (the same procedure as described in paragraph 3.2.1.2).

3.2.3.2. $^1$H NMR

The $^1$H NMR spectrum was recorded in CDCl$_3$ with a Gemini 300 MHz spectrometer. All protons are from the pHMPAmDL block except for the methylene protons, which are from PEG. From Soga et al. (2005), the values for the chemical shifts ($\delta$) given in ppm relative to TMS are known: $\delta$ 6.5 (CO-NH-CH$_2$), 5.0 (NH-CH$_2$-CH(CH$_3$)-O and CO-CH(CH$_3$)-O), 4.4 (CO-CH(CH$_3$)-OH), 3.6 (PEG methylene protons, O-CH$_2$-CH$_2$), 3.4 (b, NH-CH$_2$-CH(CH$_3$)), 2.0-0.6 (the rest of the protons are from the pHMPAmDL block).
The number of HPMAmDL units in the polymers was determined from the ratio of the integral of the methine proton (CO-CH(CH₃)-OH) at 4.4 ppm of HPMAmDL to the integral of one PEG proton at 3.6 ppm. From the resulting number of units, the number average molecular weight of the polymer was calculated (Soga et al., 2004).

3.2.3.3. Gel permeation chromatography

The molecular weights and their distribution of the block copolymers were determined by GPC. Based on the GPC calibration curve, obtained by a set of well-characterized PEG standards, it is possible to determine the peak average molecular weight (Mₚ), number average molecular weight (Mₙ), weight average molecular weight (M₇) and the polydispersity of the polymers. M₇/Mₙ refers to the polydispersity (polydispersity index, PDI), which indicates the distribution of individual molecular masses in a sample of polymers. M₇ and Mₙ are the same when the polymers are homodisperse. The PDI has a value always higher than one, but as the polymer chains approach uniform chain length, the PDI is very close to one (Saito et al., 2004).

The GPC was carried out in the same way and with the same conditions as in paragraph 3.2.2.3.

3.2.3.4. Determination of the cloud point

The determination of the CP was carried out on a Shimadzu UV-2450 with the Tm analysis software, coupled to a Shimadzu Temperature Controller. The polymers were dissolved overnight at 0°C in 120 mM ammonium acetate buffer at a concentration of 3 mg/ml. At this low temperature, the polymers are hydrated, and hence water soluble. The ammonium acetate buffer is necessary to get an aqueous solution with a pH 5, which minimizes the hydrolysis of lactate ester side groups of HPMAmDL, and guarantees the stability of the polymer.
The UV absorbance was measured at 625 nm every 0.2 °C during heating to a temperature of 15 °C. A graph was made by plotting the absorbance against the temperature. The value on the X-axis, obtained by extrapolation of this curve to absorbance zero, is considered as the CP. A sudden increase in absorbance is referred to the beginning of micelle formation (Zhu et al., 2000).

3.2.4. Micelle formation

The block copolymers were dissolved at a concentration of 0.5 mg/ml in ammonium acetate buffer (pH 5.0, 120 mM). A concentration of 0.5 mg/ml is above the CMC, which is about 0.015 mg/ml for these polymers (Soga et al., 2004)

This polymer solution was incubated overnight at 0°C, and then quickly heated to 50 °C for one minute under vigorous stirring. Afterwards, the mixture was slowly cooled down to room temperature and filtered through a 0.45 µm filter to remove possible aggregates. A schematic representation of the formation of micelles is shown in figure 3.8.

In the further reaction steps of the micelles with nanobody, only 5% of the polymers in the micelles have to be linked with nanobodies. Hence, besides the micelles only made of PDP-polymers, micelles were also made of 5% PDP modified polymers and 95% polymers without PDP moieties (total concentration of 10 mg/ml in ammonium acetate buffer, pH 5.0, 120 mM).

![Figure 3.8](image-url)
3.2.4.1. Determination of the activated disulfide groups on the micelles

The micelle samples were treated with DTT as described above (paragraph 3.2.1.2) to check if the PDP moieties were still present. The amount of released pyridine-2-thione, which absorbs at 343 nm, is equivalent to the contents of PDP groups at the outer layer of the micelles. So, the presence of polymers with activated end groups could be calculated, using equation (1).

3.2.4.2. Characterization of empty thermosensitive polymeric micelles

Dynamic Light Scattering (DLS) was used to determine the size and the size distribution (expressed as PDI) of the two kinds of micelles. At first, the DLS instrument was standardized by measuring a polystyrene latex standard.

The equipment consisted of a Malvern CGS-3 multi-angle goniometer (Malvern Ltd, Malvern, U.K.), with a He-Ne JDS Uniphase laser (λ 632.8 nm, 22 mW output power), an optical fibre-based detector, a digital LV/LSE-5003 correlator, and a temperature controller (Julabo Waterbath). Time correlation functions were analysed using the ALV-60x0 software V.3.X provided by Malvern. The diffusion coefficients calculated from the measured autocorrelation functions were related to the hydrodynamic radius of the particles via the Stokes-Einstein equation (equation (4)). All measurements were performed at an angle of 90° and at 25°C.

\[
R_h = \frac{kT}{6\pi \eta D}
\]

(4)

where: 
- \( R_h \): the hydrodynamic radius of the particles (m)
- \( k \): the Boltzmann constant (J/K)
- \( T \): the absolute temperature (K)
- \( \eta \): the viscosity of the solvent (kg m\(^{-1}\) s\(^{-1}\))
- \( D \): diffusion coefficient (m\(^2\)/s)
3.2.5. Coupling of anti-EGFR nanobodies on the micelles

3.2.5.1. Deprotection of SATA-modified nanobodies.

For active targeting, the nanobodies EGa1 against the EGFR, were linked to the micelles. Since only thiol containing molecules can be linked to the reactive PDP moieties present on the termini of the block copolymers, EGa1 was first modified in order to introduce protected sulfhydryl groups, by means of reaction with SATA. This was already done by Oliviera in 2008, synthesized as described by Duncan et al. (1983). From mass spectrometry it was calculated that there were 3-4 protected SH groups per nanobody. Next, to obtain a free sulfhydryl group, an easy deprotection step with a deacetylation solution was performed, generating a thioacetylated peptide which is reactive towards the protected PDP-polymers (figure 3.9.). In detail, 20 µl of deacetylation buffer with a pH between 7.2 – 7.5, containing 0.5 M hydroxylamine.HCl and 25 mM EDTA in PBS, was added to 200 µl of SATA modified nanobodies (1 mg/ml in PBS) and was incubated for two hours.

FIGURE 3.9.: 1. SATA DERIVATIZATION OF THE NANOBODY 2. DEPROTECTION OF THE SATA MODIFIED PROTEIN, USING A DEACETYLATION BUFFER CONTAINING 0.5M HYDROXYLAMINE.HCl AND 25 mM EDTA (http://www.piercenet.com).
3.2.5.2. Coupling of deprotected SATA-nanobodies on PDP modified micelles

The reactive sulfhydryl containing nanobodies can be conjugated to PDP-polymers to form a polymer-protein conjugate. The reaction involves the cleavage of pyridine-2-thione, as shown in figure 3.10, which absorbs UV light with a wavelength of 343 nm.

\[
\begin{align*}
\text{pHPMAmDL-} & \text{-b-(PEG-PDP)} + \text{deprotected SATA-nanobody} \\
\rightarrow \text{polymer-protein conjugate} & + \text{pyridine-2-thione}
\end{align*}
\]

\text{FIGURE 3.10.: COUPLING OF DEPROTECTED SATA MODIFIED NANOBODY TO PDP-POLYMER. IN THIS REACTION, PYRIDINE-2-THIONE IS CLEAVED.}

\text{=} \text{NANOBODY,} \quad \text{=} \text{pHPMAmDL-b-PEG}

The desired molar ratio of SH groups of the nanobody to SH groups of the polymer are: 0.25:1, 0.5:1, 1:1 and 2:1. Based on the results obtained in paragraph 3.2.1.2. and by using equation (2), the moles PDP groups per gram PDP polymers and subsequently per milligram total polymer in the solution could be calculated, using equation (5). So, it was possible to calculate the amount of nanobody (µg) and total polymer (mg) that was needed for each ratio. An example of the calculations (for the highest ratio) is given in table 3.1.

\[
\frac{[PDP]}{[\text{polymer}]} = \text{mol SH groups / g PDP polymer} \tag{5}
\]

where: [PDP]: concentration of PDP groups (mol/L)  
         [polymer]: concentration of the PDP-polymer (g/ml)
TABLE 3.1.: COUPLING OF NANOBODIES ON MICELLES: CALCULATIONS TO GET A MOLAR RATIO OF 2:1 MOLES SH GROUPS OF NANOBODIES PER MOLE SH GROUPS OF PDP-POLYMER.

<table>
<thead>
<tr>
<th>ratio 2:1, total volume 2 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>mole SH/mg PDP polymer</td>
</tr>
<tr>
<td>mole SH/mol nanobody</td>
</tr>
<tr>
<td>Mw nanobody (g/mole)</td>
</tr>
<tr>
<td>nmole SH nanobody/nmole PDP polymer</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>nanobody</th>
<th>polymer</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmole SH nanobody</td>
<td>10</td>
</tr>
<tr>
<td>nmole nanobody</td>
<td>3.33</td>
</tr>
<tr>
<td>mass nanobody (µg)</td>
<td>50.00</td>
</tr>
<tr>
<td>[nanobody] (µg/ml)</td>
<td>25.00</td>
</tr>
<tr>
<td>$V_{\text{nanobody}}$ (1mg/ml) (µL)</td>
<td>50.00</td>
</tr>
</tbody>
</table>

- a calculations were based on equation (2) and (5)
- b from mass spectrometry it was calculated that there were 3-4 protected SH groups per nanobody
- c nmol SH groups of the nanobody divided by 3 (there are 3 mole SH groups per mole nanobody)
- d nmole nanobody x Mw of the nanobody = mass of nanobody
- e mass nanobody/total volume (2ml, $V_{\text{micelles}}$) = concentration of the nanobody
- f $[\text{nanobody}] \times V_{\text{micelles}}$ = volume of nanobody
- g nmole SH groups polymer/ moles SH groups per mg PDP polymer = mass PDP polymer
- h mass of the polymers in the total solution (5% PDP-polymers and 95% unmodified polymers)
- i mass total polymer / total volume ($V_{\text{micelles}}$) = [total polymer]

A stock of polymer solution was made at a total polymer concentration of 1.3 mg/ml. Only 5% of the polymers, which form the micelles, have to be coupled with a nanobody. So, only 5% of these polymers were PDP-polymer (0.07 mg/ml). The activated nanobodies were incubated with the polymers overnight at room temperature.
3.2.5.3. Determination of the reaction progress after coupling of nanobodies on the PDP modified micelles

To check if the reaction effectively took place, the absorbance at 343 nm of the polymer-protein samples with different ratio’s was measured compared to a blank (polymer solution composed of 5% PDP polymers and 95% polymers without PDP groups in AmAc buffer). The amount of released pyridine-2-thione, which absorbs at 343 nm, is equivalent to the contents of nanobodies attached on pHPMAmDL-b-PEG-PDP. By calculating this concentration of PDP groups (based on equation (2)) and by setting the concentration of PDP groups in the sample without nanobodies at 100%, an attempt to measure the reaction progress in each sample with different amounts of nanobodies was made.

3.2.5.4. Characterization of the micelles coupled with nanobodies

Before measuring the diameter of micelles by DLS, a purification step is necessary for further experiments and application in cells. The non-coupled nanobodies and other small molecules, such as pyridine-2-thione, were removed by two times washing with ammonium acetate buffer and ultrafiltration (4000g, 20 minutes) using Vivaspin tubes with a molecular weight cut-off of 100 kDa.
4. RESULTS

4.1. DERIVATIZATION OF HO-PEG$_{5000}$-NH$_2$

4.1.1. Thiolation of HO-PEG$_{5000}$-NH$_2$ by means of SPDP

The yield of modification of HO-PEG-NH$_2$ by means of SPDP was calculated, by weighing the total mass of HO-PEG-PDP after freeze drying, typically being 80-90% (table 4.1.).

<table>
<thead>
<tr>
<th>molar ratio</th>
<th>Mw (g/mole)</th>
<th>mass (mg)</th>
<th>n (mmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO-PEG$_{5000}$-NH$_2$</td>
<td>1</td>
<td>5,033</td>
<td>500.0</td>
</tr>
<tr>
<td>SPDP</td>
<td>2</td>
<td>312</td>
<td>62.07</td>
</tr>
<tr>
<td>HO-PEG-PDP</td>
<td></td>
<td>5,230</td>
<td>468</td>
</tr>
</tbody>
</table>

% of modified HO-PEG$_{5000}$-NH$_2$: 90.07

4.1.2. DTT assay

The difference in UV absorbance at 343nm of a HO-PEG-PDP solution (0.5 mg/ml in PBS) before and after addition of DTT is given in table 4.2. After

<table>
<thead>
<tr>
<th>sample</th>
<th>$A_1$</th>
<th>$A_2$</th>
<th>$A_1 - A_1,blank$</th>
<th>$A_2 - A_2,blank$</th>
<th>$\Delta A$</th>
<th>molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>blank</td>
<td>-0.004</td>
<td>-0.009</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.018</td>
<td>0.713</td>
<td>0.0212</td>
<td>0.722</td>
<td>0.70</td>
<td>0.87</td>
</tr>
<tr>
<td>2</td>
<td>0.019</td>
<td>0.713</td>
<td>0.0222</td>
<td>0.722</td>
<td>0.70</td>
<td>0.87</td>
</tr>
<tr>
<td>3</td>
<td>0.020</td>
<td>0.720</td>
<td>0.0232</td>
<td>0.728</td>
<td>0.71</td>
<td>0.87</td>
</tr>
</tbody>
</table>

$^a$ UV-absorbance at a wavelength of 343 nm before DTT was added.

$^b$ UV-absorbance at a wavelength of 343 nm fifteen minutes after DTT was added.

$^c$ difference in absorbance before and after DTT was added [(A$_2$ - A$_1,blank$) - (A$_1$ - A$_2,blank$)].

$^d$ the molar ratio of SPDP versus HO-PEG$_{5000}$-NH$_2$ was calculated using equation (1).
repeating this experiment, reproducible results were obtained being typically between 0.85 and 0.90 moles SPDP per moles PEG.

4.2. SYNTHESIS OF THE MACROINITIATOR

4.2.1. The DCC coupling method

After freeze drying of the filtrated macroinitiator solution, the weight was determined to estimate the yield of this synthesis (table 4.3). This yield was usually between 80 - 90%.

<table>
<thead>
<tr>
<th>molar ratio</th>
<th>Mw (g/mole)</th>
<th>mass (g)</th>
<th>n (mmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO-PEG-PDP</td>
<td>2</td>
<td>5,230</td>
<td>0.612</td>
</tr>
<tr>
<td>(PDP-PEG)₂-ABCPA</td>
<td>1</td>
<td>10,704</td>
<td>0.230</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>yield of the synthesis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>86.05</td>
</tr>
</tbody>
</table>

4.2.2. \(^1\text{H} \) NMR

4.2.2.1. \(^1\text{H} \) NMR of the macroinitiator before addition of TAIC

A \(^1\text{H} \) NMR spectrum of the macroinitiator is shown in figure 4.1. At \(\delta \) 3 – 4, a big peak of the PEG protons is clearly visible. The \(^1\text{H} \) signals of the protons of ABCPA at \(\delta \) 1.63 - 1.7 and 2.2 - 2.6 have respectively an integral of 5.58 and 2.71. The molar ratio of PEG : ABCPA was calculated to be 1.7, using equation (3).
4.2.2.2. $^1$H NMR of the macroinitiator after addition of TAIC

In figure 4.2, the spectrum of the macroinitiator after the addition of TAIC is shown. In this spectrum, there is a small peak at δ 4.4 with an integral of 0.16, which was not present in the spectrum without TAIC. This peak is caused by the downfield OH-shift, due to the derivatization of the hydroxyl group by means of TAIC. Consequently, it was possible to calculate the percentage of unreacted PEG-OH (HO-PEG-PDP or the starting compound HO-PEG-NH$_2$). Since the integral of a peak refers to the number of protons, the integral at δ 4.4 would be two if there would be only unreacted PEG-OH (two $\alpha$ protons next to the OH group). In this spectrum, the integral is 0.16, so it can be decide that there are 8% unreacted PEG-OH molecules.

![Figure 4.1: $^1$H NMR of the macroinitiator before addition of TAIC. At 1.63 + 1.7 + 2.2-2.6 ppm are the $^1$H-signals of the protons of ABCPA.](image-url)
4.2.3. Gel permeation chromatography

The peak average molecular weight and the elution time of the molecules present in the macroinitiator sample are visualized in a gel permeation chromatogram (figure 4.3). Table 4.4 shows that circa 37% of the molecules in the reaction mixture have a $M_p$ of about 11,000 g/mole (retention time 20.5 min.) and 43% have a $M_p$ of 5,800 g/mole (retention time 21.8 min.). Other small molecules elute at 19.5, 25 and 26.5 minutes.
FIGURE 4.3: GPC CHROMATOGRAM OF THE MACROINITIATOR.

TABLE 4.4.: THE GPC RESULTS OF THE MACROINITIATOR.

<table>
<thead>
<tr>
<th>retention time (min)</th>
<th>(M_p) (g/mole)</th>
<th>area</th>
<th>% area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19.8</td>
<td>17 134</td>
<td>6 822 434</td>
</tr>
<tr>
<td>2</td>
<td>20.5</td>
<td>11 493</td>
<td>20 200 275</td>
</tr>
<tr>
<td>3</td>
<td>21.8</td>
<td>5 813</td>
<td>24 107 696</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>886</td>
<td>1 021 268</td>
</tr>
<tr>
<td>5</td>
<td>26.3</td>
<td>347</td>
<td>2 982 362</td>
</tr>
</tbody>
</table>

4.3. BLOCK COPOLYMER SYNTHESIS

4.3.1. Radical polymerization

To estimate roughly the yield of polymerization, the unreacted monomers after centrifugation and evaporation were weighed. The weight of unreacted HPMAmDL was usually about 100 mg. So, the yield of polymerization was about 73%, because table 4.5 shows that there were 27% unreacted monomers.
TABLE 4.5.: THE YIELD OF POLYMERIZATION

<table>
<thead>
<tr>
<th></th>
<th>Mw (g/mole)</th>
<th>mass (mg)</th>
<th>n (mmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPMAmDL start</td>
<td>287</td>
<td>411.0</td>
<td>14.32</td>
</tr>
<tr>
<td>HPMAmDL end</td>
<td>287</td>
<td>110.9</td>
<td>0.3864</td>
</tr>
<tr>
<td>percentage unreacted HPMAmDL (%)</td>
<td></td>
<td></td>
<td>26.98</td>
</tr>
</tbody>
</table>

4.3.2. $^1$H NMR

The average number of HPMAmDL units per block copolymer was determined as described above (paragraph 3.2.3.2.) based on the $^1$H NMR spectrum shown in figure 4.4. At $\delta$ 4.4 there is a peak of methine protons of the polymer (CO-CH(CH$_3$)-OH) with an integral of 61.49. This means that there are about 60 units of HPMAmDL, with a molecular weight of 287, per molecule PEG. Hence, the $M_n$ of the diblock copolymer is approximately 17,507 g/mole.
4.3.3. Gel permeation chromatography

The chromatogram of the polymer can be seen figure 4.5. Table 4.6 shows that almost 80% of the molecules have a Mn of about 34,000 g/mole (retention time 18.1 min.). Two other peaks of smaller molecules are also present, having respectively a molecular weight of 10,000 and 5,000 g/mole.

![GPC Chromatogram of the Polymer pHMAmDL-b-PEG](image)

**TABLE 4.6. THE GPC RESULTS OF THE POLYMER pHMAmDL-b-PEG**

<table>
<thead>
<tr>
<th></th>
<th>$R_t^a$ (min)</th>
<th>$M_n$ (g/mole)</th>
<th>$M_w$ (g/mole)</th>
<th>$M_p$ (g/mole)</th>
<th>$M_z$ (g/mole)</th>
<th>PDI $^b$</th>
<th>area</th>
<th>% area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18.1</td>
<td>31 956.4</td>
<td>4 5871.5</td>
<td>34 659.7</td>
<td>67 802.7</td>
<td>1.4</td>
<td>47 532 937</td>
<td>78.27</td>
</tr>
<tr>
<td>2</td>
<td>20.3</td>
<td>6 938.6</td>
<td>7 965.6</td>
<td>10 330.5</td>
<td>8 946.5</td>
<td>1.1</td>
<td>13 191 600</td>
<td>21.72</td>
</tr>
<tr>
<td>3</td>
<td>21.5</td>
<td>4 944.0</td>
<td>5 074.5</td>
<td>5 156.7</td>
<td>5 199.9</td>
<td>1.0</td>
<td>5 546 646</td>
<td>9.13</td>
</tr>
</tbody>
</table>

$^a$ $R_t$: Retention time

$^b$ PDI: polydispersity index

4.3.4. Determination of the cloud point

Measuring of the UV absorbance at 625 nm every 0.2 °C during heating the polymer solution up to 15°C, results in the following graph (figure 4.6). The CP of the polymer is at 4°C, visualized by the sudden increase in UV absorbance.
FIGURE 4.6: THE DETERMINATION OF THE CLOUD POINT AF AN AQUEOUS POLYMER SOLUTION BY MEASURING THE UV ABSORBANCE AT 625 NM (Y-AXIS) EVERY 0.2 °C DURING HEATING TO 15°C. THE VALUE ON THE X-AXIS (TEMPERATURE, °C) OBTAINED BY EXTRAPOLATION OF THE CURVE TO ABSORBANCE ZERO IS CONSIDERED AS THE CP.

4.4. MICELLE FORMATION

4.4.1. Determination of the activated disulfide groups on the micelles

4.4.1.1. DTT assay on micelles composed of 100% PDP-polymers

TABLE 4.7: UV-ABSORBANCE OF A MICELLE SOLUTION AT 343 NM (MICELLES COMPOSED OF 100% PDP-POLYMERS, 0.5 MG/ML) BEFORE AND AFTER DTT ADDITION, COMPARED TO A BLANK (AMMONIUM ACETATE BUFFER). THE SAMPLE WAS MEASURED IN TRIPLICATE.

<table>
<thead>
<tr>
<th>sample</th>
<th>$A_1$</th>
<th>$A_2$</th>
<th>$A_1 - A_{1,\text{blank}}$</th>
<th>$A_2 - A_{2,\text{blank}}$</th>
<th>$A_1 - A_{1,\text{blank}}$ - $A_2 - A_{2,\text{blank}}$</th>
<th>molar ratio$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>blank</td>
<td>-0.007</td>
<td>-0.016</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.119</td>
<td>0.256</td>
<td>0.1256</td>
<td>0.272</td>
<td>0.146</td>
<td>0.81</td>
</tr>
<tr>
<td>2</td>
<td>0.109</td>
<td>0.246</td>
<td>0.1156</td>
<td>0.262</td>
<td>0.147</td>
<td>0.82</td>
</tr>
<tr>
<td>3</td>
<td>0.102</td>
<td>0.243</td>
<td>0.1086</td>
<td>0.259</td>
<td>0.150</td>
<td>0.84</td>
</tr>
</tbody>
</table>

$^a$ UV-absorbance at a wavelength of 343 nm before DTT was added.

$^b$ UV-absorbance at a wavelength of 343 nm fifteen minutes after DTT was added.

$^c$ the difference in absorbance before and after DTT was added \([A_2 - A_{1,\text{blank}}] - [A_1 - A_{2,\text{blank}}]\).

$^d$ the molar ratio of SPDP versus OH-PEG$_{5000}$-NH$_2$ was calculated using equation (1).
From table 4.7 it can be concluded that almost 80% of the polymers in the micelles were still functionalized with PDP moieties, based on equation (1).

4.4.1.2. DTT assay on micelles composed of 5% PDP polymers and 95% unmodified polymers

The results in table 4.8 indicate that the majority of polymer chains were still functionalized with SPDP.

TABLE 4.8.: UV ABSORBANCE OF A MICELLE SOLUTION (MICELLES COMPOSED OF 5% PDP-POLYMERS AND 95% UNMODIFIED POLYMERS, TOTAL CONCENTRATION OF 10 MG/ML), BEFORE AND AFTER DTT WAS ADDED, COMPARED TO A BLANK (AMMONIUM ACETATE BUFFER). THE SAMPLE WAS MEASURED IN TRIPLICATE.

<table>
<thead>
<tr>
<th>sample</th>
<th>A₁ ²</th>
<th>A₂ ²</th>
<th>A₁-X₁,blank</th>
<th>A₂-X₂,blank</th>
<th>ΔA ³</th>
<th>molar ratio ²⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>blank</td>
<td>-0.008</td>
<td>-0.013</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.014</td>
<td>1.132</td>
<td>1.022</td>
<td>1.145</td>
<td>0.123</td>
<td>0.038</td>
</tr>
<tr>
<td>2</td>
<td>1.012</td>
<td>1.126</td>
<td>1.020</td>
<td>1.139</td>
<td>0.119</td>
<td>0.037</td>
</tr>
<tr>
<td>3</td>
<td>1.015</td>
<td>1.130</td>
<td>1.023</td>
<td>1.143</td>
<td>0.120</td>
<td>0.037</td>
</tr>
</tbody>
</table>

² UV-absorbance at a wavelength of 343 nm before DTT was added.
³ UV-absorbance at a wavelength of 343 nm fifteen minutes after DTT was added.
⁴ the difference in absorbance before and after DTT was added \([A₂ - A₁,blank] - (A₁ - A₂,blank)\].
²⁴ the molar ratio of SPDP to polymer was calculated using equation (1).

4.4.1.3. The characterization of empty micelles

The size \((Z_{\text{average}})\) and the PDI of the micelles, composed of 100% PDP-polymers or 5% PDP polymers, determined by DLS, are shown in table 4.9. The distribution of the size of the nanoparticles is demonstrated in figure 4.7 and 4.8, by plotting the intensity (%) against the logarithm of the size of the micelles (nm).
TABLE 4.9: THE SIZE OF THE MICELLES AND THEIR DISTRIBUTION (PDI), DETERMINED BY DLS.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$Z_{\text{average}}$ (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% PDP $^a$</td>
<td>96.3</td>
<td>0.151</td>
</tr>
<tr>
<td>5% PDP $^b$</td>
<td>63.54</td>
<td>0.069</td>
</tr>
</tbody>
</table>

$^a$ Sample with micelles composed of 100% PDP-polymers
$^b$ Sample with micelles composed of 5% PDP-polymers and 95% polymers without PDP groups.
$^c$ The average diameter of all particles in the sample
$^d$ PDI: polydispersity index of the micelles

**FIGURE 4.7.:** THE SIZE DISTRIBUTION OF THE MICELLES COMPOSED OF 100% PDP-POLYMERS.

**FIGURE 4.8.:** THE SIZE DISTRIBUTION OF THE MICELLES COMPOSED OF 5% PDP-POLYMERS AND 95% POLYMERS WITHOUT PDP MOIETIES.
4.5. COUPLING OF ANTI-EGFR NANOBODIES ON THE MICELLES

4.5.1. Determination of the reaction progress after coupling of nanobodies on the PDP modified micelles

In the polymer solution containing 5% PDP-polymers and 95% polymers without PDP moieties, the average concentration of the PDP groups was 4.8 µM (table 4.10). This calculation was based on the law of Lambert-Beer (equation (3)).

To calculate the yield of the reaction, the PDP concentration on the micelles with no added nanobody (table 4.10, measured with the DTT assay), was set as 100%. The reaction progress (expressed as percentage of reaction) of the nanobody coupled in each sample with different ratio of moles SH groups of nanobody to moles SH groups of polymers was calculated. An example of the results (of the highest ratio of SH groups of nanobody to SH groups of polymer, 2:1) are shown in table 4.11.

<table>
<thead>
<tr>
<th>sample</th>
<th>$A_1^a$</th>
<th>$A_1^a - A_{1,\text{blank}}$</th>
<th>$A_2^b$</th>
<th>$A_2^b - A_{2,\text{blank}}$</th>
<th>$\Delta A^c$</th>
<th>[PDP] (µM)$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>blank</td>
<td>0.087</td>
<td>0.090</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$0_A^e$</td>
<td>0.234</td>
<td>0.147</td>
<td>0.276</td>
<td>0.187</td>
<td>0.040</td>
<td>4.950</td>
</tr>
<tr>
<td>$0_B^f$</td>
<td>0.213</td>
<td>0.126</td>
<td>0.253</td>
<td>0.164</td>
<td>0.038</td>
<td>4.641</td>
</tr>
</tbody>
</table>

average [PDP] (µM) 4.796

$^a$ UV-absorbance at a wavelength of 343 nm before DTT was added.

$^b$ UV-absorbance at a wavelength of 343 nm fifteen minutes after DTT was added.

$^c$ the difference in absorbance before and after DTT was added $[(A_2 - A_{1,\text{blank}}) - (A_1 - A_{2,\text{blank}})]$.

$^d$ the concentration of PDP-groups in the polymer solution, calculated using equation (3).

$^e,f$ two micelle samples without nanobodies

<table>
<thead>
<tr>
<th>sample</th>
<th>$\Delta A$</th>
<th>[PDP] (µM)</th>
<th>reaction progress (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A</td>
<td>0.0137</td>
<td>1.691</td>
<td>35.24</td>
</tr>
<tr>
<td>2B</td>
<td>0.0525</td>
<td>6.498</td>
<td>135.36</td>
</tr>
<tr>
<td>2C</td>
<td>0.0285</td>
<td>3.527</td>
<td>73.48</td>
</tr>
</tbody>
</table>

* EGa1-micelle sample with a ratio of moles SH-groups of the nanobody to moles SH-groups of the polymer (PDP) of 2:1. The sample was measured in triplicate (A-B-C).

* difference in absorbance at 343 nm between the protein-micelle sample and a micellar sample without nanobodies.

* concentration of PDP groups in de micelle-protein solution (µM)

* the extent to which the reaction is continued, expressed as percentage.

4.5.2. Characterization of the micelles coupled to nanobodies.

The size of the micelles ($Z_{\text{average}}$) and their distribution (PDI) is shown in table 4.12. The micelle samples without nanobodies and the EGa1-micelle samples with different ratios of moles SH-groups of nanobodies per mole SH-groups of PDP-polymer, have a size of 60-70 nm and a monomodal distribution (one narrow peak in the statistic size distribution).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Z\text{average} (nm)</th>
<th>PDI $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0\textsubscript{A}</td>
<td>66.18</td>
<td>0.052</td>
</tr>
<tr>
<td>0\textsubscript{B}</td>
<td>74.93</td>
<td>0.259</td>
</tr>
<tr>
<td>0\textsubscript{C}</td>
<td>69.42</td>
<td>0.11</td>
</tr>
<tr>
<td>0.25\textsubscript{A}</td>
<td>69.26</td>
<td>0.169</td>
</tr>
<tr>
<td>0.25\textsubscript{B}</td>
<td>73.78</td>
<td>0.181</td>
</tr>
<tr>
<td>0.25\textsubscript{C}</td>
<td>72.55</td>
<td>0.177</td>
</tr>
<tr>
<td>0.5\textsubscript{A}</td>
<td>70.79</td>
<td>0.198</td>
</tr>
<tr>
<td>0.5\textsubscript{B}</td>
<td>71.93</td>
<td>0.238</td>
</tr>
<tr>
<td>0.5\textsubscript{C}</td>
<td>71.06</td>
<td>0.177</td>
</tr>
<tr>
<td>1\textsubscript{A}</td>
<td>67.96</td>
<td>0.238</td>
</tr>
<tr>
<td>1\textsubscript{B}</td>
<td>74.7</td>
<td>0.211</td>
</tr>
<tr>
<td>1\textsubscript{C}</td>
<td>69.56</td>
<td>0.217</td>
</tr>
<tr>
<td>2\textsubscript{A}</td>
<td>73.67</td>
<td>0.196</td>
</tr>
<tr>
<td>2\textsubscript{B}</td>
<td>68.54</td>
<td>0.179</td>
</tr>
<tr>
<td>2\textsubscript{C}</td>
<td>69.74</td>
<td>0.125</td>
</tr>
</tbody>
</table>

$^a$ the ratio of moles SH groups of nanobody per moles SH groups of polymers were: 0.25:1, 0.5:1, 1:1 and 2:1. Each sample was measured in triplicate (A-B-C)

$^b$ PDI: polydispersity index of the micelles in each sample
5. DISCUSSION

5.1. DERIVATIZATION OF HO-PEG\textsubscript{5000}-NH\textsubscript{2}

5.1.1. Thiolation of HO-PEG\textsubscript{5000}-NH\textsubscript{2} by means of SPDP

The synthesis of PDP-functionalized polymers was based on a previously used similar route to synthesize bocylated polymers. Rijcken (2007, chapter 9) used a twofold excess of SPDP versus bocylated HO-PEG-NH\textsubscript{2}. When the thiolation in this thesis was also performed in a molar ratio HO-PEG-PDP versus SPDP of 1:2, and on a scale about 300 - 500 mg, reproducible results were obtained.

A higher ratio of 1:2.5 was tried too, which gave the same range of yield of modification (between 80-90%). Given the cost of both products, a ratio of 1:2 seemed most suitable. Using a molar ratio of 1:2, however, could result in more free reactive NH\textsubscript{2} groups that might react competitively in the synthesis of the macroinitiator (see below, 5.2.3).

5.1.2. DTT assay

From the obtained results it can be concluded that around 90% of the HO-PEG-NH\textsubscript{2} molecules were coupled with SPDP, and 10% of SPDP did not participate in the reaction. The free SPDP molecules were removed by the PD-10 column.

This experiment was done on each HO-PEG-PDP batch and gave every time similar results between 0.85 and 0.90 moles SPDP per moles PEG. As expected, these results are almost the same as in paragraph 5.1.1. Rijcken (2007) recovered almost 90% of the PDP moieties present on the polymers after treatment with DTT.
5.2. SYNTHESIS OF MACROINITIATOR

5.2.1. The DCC coupling method

The novel macroinitiator was obtained in a good yield (± 80 – 90 %), but one should keep in mind that this is based on the total amount of product after freeze drying, which consists of desired bissubstituted ABCPA but also other side products, such as monosubstituted ABCPA.

5.2.2. $^1$H NMR

From the $^1$H NMR spectrum before the addition of TAIC it was calculated that there are on average 1.7 molecules PEG-PDP grafted to one molecule ABCPA. This means that there are the desired bisPEGylated macroinitiator but also some PDP-PEG-ABCPA molecules. The latter can be caused by the reaction of the hydroxyl group of water with the active ester from the DCC coupling. Due to the presence of PDP-PEG-ABCPA, homopolymers of pHPMAmDL can be formed in the polymerization reaction. These homopolymers, being hydrophobic, cause a decrease of the overall cloud point of a polymer solution.

The $^1$H NMR spectrum of the macroinitiator after the addition of TAIC, indicates that most of the HO-PEG-PDP molecules reacted with the active ester. Only 8% of the molecules in the reaction mixture is PEG-OH (HO-PEG-PDP or the starting compound HO-PEG-NH$_2$). This result was low enough to use this macroinitiator for polymerization. In the GPC results, 8% of the compounds with a $M_p$ of 5,000 g/mole will be PEG-OH.
5.2.3. **Gel permeation chromatography**

GPC analysis shows a trimodal molecular weight distribution of the macroinitiator. The second peak with a retention time of 20.5 minutes, corresponding to a molecular weight of about 11,000 g/mole, is ascribed to the presence of biPEGylated ABCPA. So, the sample contains 37% of the desired macroinitiator. However, 44% of the product is a compound with a molecular weight of 5,800 g/mole (retention time of 21.7 min.), which refers to monosubstituted ABCPA as well as unreacted PEG (HO-PEG-PDP or HO-PEG-NH₂). From the information of the NMR, it is possible to discriminate between these two products. In paragraph 5.2.2., it was calculated that there is almost 8% unreacted PEG-OH. Hence, 35% of the compounds with a $M_p$ of 5,800 g/mole belongs to unwanted PDP-PEG-ABCPA. Rijcken (2007) reported around 25% of monobocylated PEG.

PDP-PEG-ABCPA is formed by the reaction of the hydroxyl group of water with the active ester from the DCC coupling. Due to the presence of monoPEGylated ABCPA, homopolymers of pHPMAmDL can be formed in the polymerization reaction. These homopolymers, being hydrophobic, cause a decrease of the overall cloud point of a polymer solution. So, the amounts of formed PDP-PEG-ABCPA has to be as low as possible. To decrease the percentage of monosubstituted ABCPA, it is necessary to take care of the presence of water. The glassware and molar sieves were dried overnight in the oven and everything was done under nitrogen atmosphere, but it is impossible to get rid of water completely.

The first peak in the chromatogram, corresponding to a retention time of 19.8 minutes and a molecular weight of 17,000 g/mole is maybe a result of the reaction of the free NH₂ groups of unreacted HO-PEG-NH₂ with ABCPA. From the treatment of HO-PEG-PDP samples with DTT, it is known that there are about 10% unreacted HO-PEG-NH₂ molecules. These unreacted molecules can form PEG-ABCPA-NH-PEG-O-ABCPA-O-PEG. In general, the OH-groups are more reactive than the NH₂-groups but this side reaction can take place.
The small peaks at a higher retention time are impurities such as traces of unreacted SPDP, DCC, ABCPA or DPTS, despite the use of a PD-column, filtering, etc.

5.3. BLOCK COPOLYMER SYNTHESIS

5.3.1. Radical polymerization

The 70%-yield of the synthesis of the thermosensitive block copolymer was similar as the yield obtained by Soga in 2006 (a yield between 50% and 70% was reported).

5.3.2. $^1$H NMR

The number average molecular weight of approximately 17,000 g/mole was as expected since Soga et al. in 2004 reported similar values for these polymers. The physical characteristics of polymers and micelles depend on the length of the hydrophobic HPMAmDL block (paragraph 5.3.4.).

5.3.3. Gel permeation chromatography

The first peak in the chromatogram with a retention time of 18.1 minutes, corresponding to a molecular weight of 34,000 g/mole, is ascribed to the block copolymer pHMAmDL-$b$-PEG-PDP. Unfortunately, using this technique or NMR, it is impossible to reveal the presence of homopolymers of pHMAmDL. The homopolymers can be hidden in the first peak. The challenge is to minimize the amount of pHMAmDL because, being hydrophobic, it could affect the overall cloud point of the polymer.

The second peak, with a retention time of 20.3 minutes, refers to the unreacted (PDP-PEG)$_2$-ABCPA molecules with a $M_p$ of 10,000 g/mole. The third
peak, eluting from the column at 21.5 minutes, is ascribed to the unreacted HO-PEG-NH₂ or PDP-PEG-ABCPA.

In the future, GPC can be associated with double RI and UV detection in order to see also the PDP groups on the polymer.

5.3.4. Determination of the cloud point

The thermosensitive block of the polymer starts to become insoluble due to the dehydration above the CP, and the polymer assembles into micelles. This is visualized by a sudden increase of the UV absorbance at 625 nm at 4°C. It is important to have a cloud point below body temperature, to ensure that micelles are obtained at 37°C. Soga reported in 2006 a CP of 6°C of a pHPMAmDL-b-PEG block copolymer with a $M_n$ of 15,000 g/mole. A decrease of the CP with increasing HPMAmDL units per molecule PEG-PDP was found, due to the hydrophobicity of the monomer. Also a decrease in the CP is observed to the inevitable presence of pHPMAmDL homopolymers (as explained in paragraph 5.2.3.).

5.4. MICELLE FORMATION

5.4.1. Determination of the activated disulfide groups on the micelles

Because there was a likelihood that the hydrophobic PDP groups should co-assemble into the hydrophobic core of the micelles, it was useful to check if the PDP groups were still on the outer part of the micelles. The results show that this assumed co-assembly did not take place, but that the majority of the PDP moieties were still present and most importantly, accessible to DTT.
5.4.2. Characterization of the empty micelles

The micelles composed of 100% PDP polymers had usually an average diameter of 60 nm, the micelles made of 5% PDP polymers and 95% unmodified polymers typically about 90 nm. Soga (2006) reported a diameter of 60 nm of unmodified micelles. It can be assumed that the size of the micelles made of 100% PDP modified polymers is bigger due to the PDP groups on the outer part of the micelles.

The size distribution of both kinds of micelles indicates a narrow and monomodal distribution. This means that the sample is monodisperse (PDI < 0.2), containing nanoparticles with a uniform size.

5.5. COUPLING OF ANTI-EGFR ANTIBODIES ON THE MICELLES

5.5.1. Determination of the reaction progress after coupling of nanobodies on the PDP modified micelles

At first sight, the results seem not reproducible at all. Because of the low concentrations used here, the UV absorbing pyridine-2-thione molecules were also present in a very low concentration. These low amounts were used because there was less than 1 ml SATA modified nanobody at our disposal to do this experiment, because this nanobody is quite expensive. So, at these low concentrations spectrophotometry is not sensitive enough to detect such a low amount of pyridine-2-thione. The measured absorbance is probably only the noise.

A better choice to detect nanobodies coupled to micelles would be the use of SDS-PAGE electrophoresis combined with Western blotting, like Oliviera (2008) performed on nanobody conjugated liposomes. Van Dijk-Wolthuis et al. (1998) also used SDS-PAGE analysis, for the determination of the conjugation efficiency of Fabs on micelles. By SDS-PAGE, after loading the EGa1-micelle samples on a polyacrylamide gel and addition of SDS, the polymer-nanobody conjugates will be separated according to size. Conjugates are expected to have a band at around
50 kDa consisting of the 35 kDa polymer and 15 kDa nanobody. In addition, a more sensitive HPLC method for pyridine-2-thione can be used in order to adequately determine the amount that is produced after conjugation.

5.5.2. Characterization of the micelles coupled to the nanobodies

The results of the DLS method for measuring the size of the nanoparticles and their distribution (PDI) do not show any difference between micelles without nanobodies and micelles coupled with nanobodies, before or after purification using ultrafiltration. This is clearly because of the low amount of nanobody present on the micelles. In addition, no increase in size of liposomes was observed after nanobody coupling to liposomes from Oliveira in 2008.

The monodispersity indicates that there was no adduct formation, which could take place by coupling of one SH group on the outside of a micelle to another SH groups coupled on the outside of another micelle. This would be possible because each of these nanobodies contains 3-4 SATA groups.
6. CONCLUSIONS

In this study, thermosensitive block copolymers were synthesized composing of a hydrophilic and a thermosensitive block, respectively PEG-PDP and HPMAmDL. The hydrophilic block contains a thiol group in order to be able to couple a nanobody on it.

At first, HO-PEG$_{5000}$-NH$_2$ was successfully functionalized with SPDP. By means of the DTT assay, it was found that 80-90% of the PEG molecules were modified with thiol groups.

It seems that the synthesis of the macroinitiator (PDP-PEG)$_2$-ABCPA is very sensitive to the presence of water. The results of the NMR and the GPC indicate that approximately 40% of the molecules in the reaction mixture were (PDP-PEG)$_2$-ABCPA but also monoPEGylated ABCPA was present, which was formed by water attacking the active ester formed in the DCC coupling reaction. Due to this molecule, homopolymers of pHPMAmDL can be formed in the polymerization step. These homopolymers, being hydrophobic, cause a decrease of the overall cloud point. So, it is important to minimize the amount of water as much as possible.

However, the pHPMAmDL-$b$-PEG-PDP block copolymers were successfully synthesized by radical polymerization using the monomer HPMAmDL and the macroinitiator (PDP-PEG)$_2$-ABCPA. GPC analysis shows a $M_n$ between 13,000-17,000 g/mole and a PDI close to one. In the future, GPC can be associated with double RI and UV detection in order to see also the PDP groups on the polymer.

The thermosensitive polymers had a cloud point far below body temperature, between 4 – 7 °C which was as expected for this type of polymers. This temperature depends on the block length of the polymer. The more hydrophobic HPMAmDL units per molecule PEG-PDP, the lower the CP will be.

When an ice-cold aqueous polymer solution composed of 5% PDP polymers and 95% unmodified polymers in ammonium acetate buffer was heated to 50°C for
one minute under vigorous stirring, stable monodisperse micelles were formed with a size of about 65 nm.

The conjugation reaction of the nanobodies to the micelles still has to be optimized. The used amounts were too low and spectrophotometry was not sensitive enough. The use of SDS-PAGE electrophoresis is one of the future plans, as well as the usage of a HPLC method for the determination of pyridine-2-thione.

Another future plan is the synthesis of fluorescence probed polymers for visualization of cellular uptake of nanoparticles. To optimize the therapeutic efficacy of the encapsulated pharmaceutical agents, it is important to study the kinetics of the \textit{in vitro} cellular uptake as well as the intracellular distribution. The trafficking of the nanoparticles and their localization into different intracellular compartments can be visualized by confocal laser scanning microscopy (CLSM) after coupling of a fluorescent label to the polymers (Maysinger \textit{et al.}, 2007). Rhodamine methacrylate (RhoMA) can be used as dye. The coupling of this fluorescent probe to these polymers was carried out before by this department (Soga \textit{et al.}, 2005; Rijcken, 2007).
7. REFERENCES


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