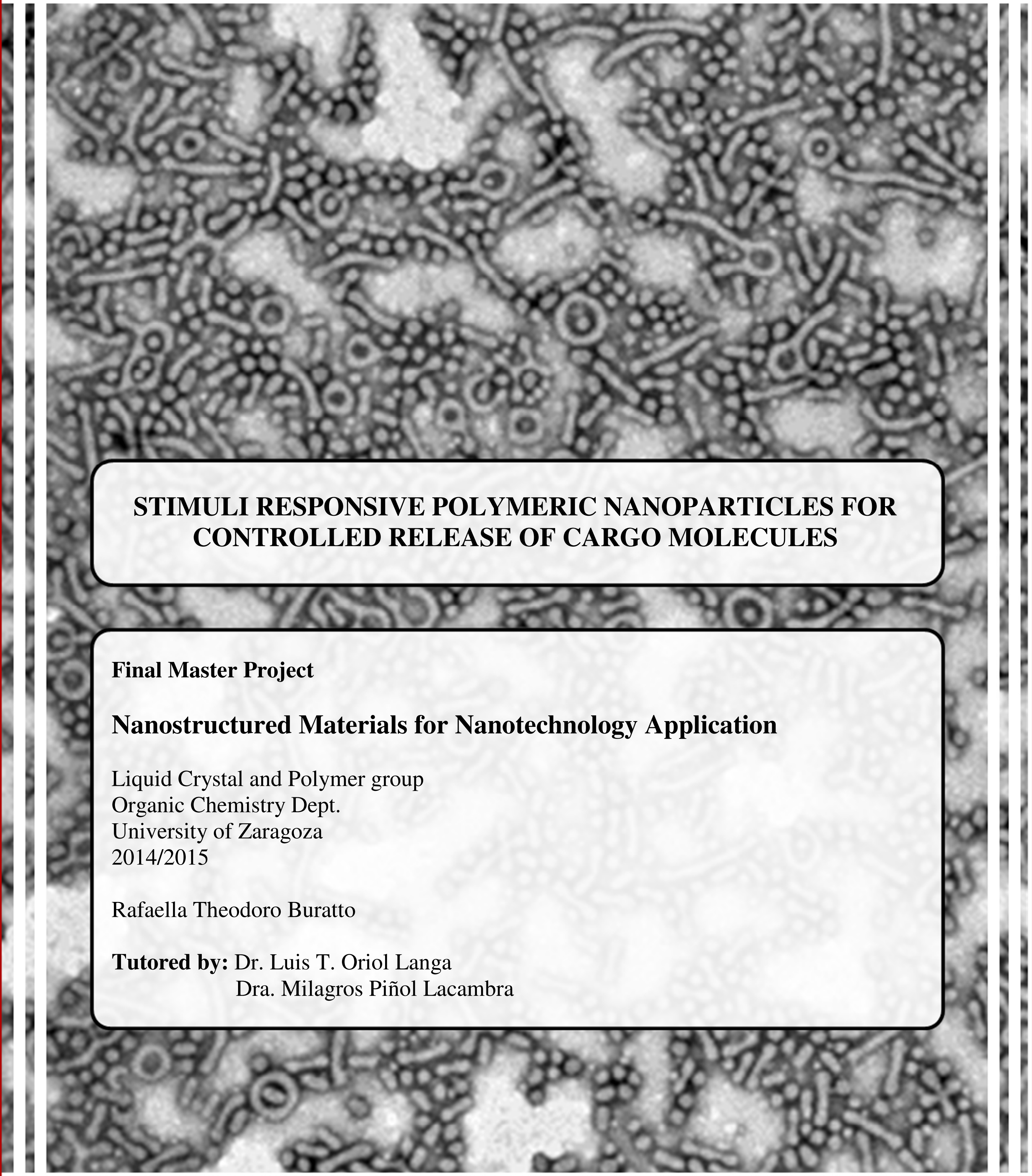
Departamento de Química Orgánica
Universidad Zaragoza

Facultad de Ciencias
Universidad Zaragoza



STIMULI RESPONSIVE POLYMERIC NANOPARTICLES FOR CONTROLLED RELEASE OF CARGO MOLECULES

Final Master Project

Nanostructured Materials for Nanotechnology Application

Liquid Crystal and Polymer group
Organic Chemistry Dept.
University of Zaragoza
2014/2015

Rafaella Theodoro Buratto

Tutored by: Dr. Luis T. Oriol Langa
Dra. Milagros Piñol Lacambra

icma
Instituto de Ciencia de Materiales de Aragón

CSIC
Consejo Superior de Investigaciones Científicas

Universidad Zaragoza

BURATTO, Rafaella Theodoro. STIMULI RESPONSIVE POLYMERIC NANOPARTICLES FOR CONTROLLED RELEASE OF CARGO MOLECULES. University of Zaragoza. Faculty of Science, Organic Chemistry department, Master in Nanostructured Materials for Nanotechnology Application, 2014/2015.

Luis T. Oriol Langa and Milagros Piñol Lacambra, official professors in Organic Chemistry department and Supervisors of the Final Master Project of Rafaella Theodoro Buratto,

TO WHOM MAY CONCIDER:

This work has been carried in the labs of the Department of Organic Chemistry in the Science Faculty of University of Zaragoza and Institute of Materials science of Aragon and in According to the final project requirement of the master "Nanostructured Materials for Nanotechnology Application"

Zaragora, 18th September 2015

Luis T. Oriol Langa

Milagros Piñol Lacambra

ABSTRACT: “STIMULI RESPONSIVE POLYMERIC NANOPARTICLES FOR CONTROLLED RELEASE OF CARGO MOLECULES”

Block copolymers (BCs) are complex macromolecules having, at least, two different polymeric chains chemically connected. These blocks are, in general, mutually immiscible, which leads to phase separation. Due to the length of the macromolecular blocks, this segregation occurs at the nanoscale. If BCs are formed by hydrophilic and hydrophobic blocks (amphiphilic BCs) these materials can undergo segregation in a selective solvent, e.g. water, resulting on micelles or vesicles above a critical concentration, as occurs with surfactants. Polymeric micelles or vesicles have higher stability than those formed by low molecular weight amphiphiles and have been explored in Medicine as nanocarriers for targeted drug delivery.

Objective of this project is the preparation of nanoparticles from amphiphilic BCs to bond stimuli-responsive molecules by multiple hydrogen bonding (molecular recognition). Hydrophobic blocks having 2,6-diacylaminopyridine moieties will be employed for supramolecular assemblies. The high tendency to form polymeric nanoparticles of these BCs will be studied. Azobenzene groups will be easily incorporated to provide a light response to the nanoparticles that can be used for the release of cargo molecules. Furthermore, the presence of pyridine rings also provide of a pH response.

Tasks to be carried out in this project initially are:

- Preparation of precursors and supramolecular amphiphilic BCs having 2,6-diacylaminopyridine groups.
- Complete characterization of the prepared materials, including spectroscopic and thermal analysis techniques.
- Preparation of polymeric nanoparticles by using different methodologies and study by transmission electron microscopy.

INDEX

ABSTRACT

1. INTRODUCTION

1.1 Copolymers Structure and Applications

Polymers are the product of the repeated union of many small molecules. Essentially, they are natural or synthetic macromolecules with high molecular mass that are formed by a reduced type of repeating units. Polymers are very important materials, fundamental to the maintenance of life as they are present in living organisms, such as proteins, cellulose and nucleic acids. Although natural polymers can be chemically modified, such as rubber, nitrocellulose or cellulose acetate (known as semisynthetic polymers), synthetic polymers can be obtained from the reaction of low molecular mass compounds known as monomers in a process called polymerization. As pioneering works in the field of semisynthetic polymers, in the 19th century Henri Braconnot used cellulose as precursor of collodion and nitrocellulose. Addition of sulfur on the natural rubber by Goodyear, called as vulcanization, led to the popularization of the first semisynthetic polymer, which became widely used. The first fully synthetic and commercial polymer is due to Baekeland at the beginning of the 20th century.

When a polymer is only constituted by one repeating unit, the macromolecule is called homopolymer. However, some polymeric structures are constituted by two or more repeating units (derived from different monomers) and these macromolecules are known as copolymers. Copolymers are a significant class of polymer that can be classified according to how the monomeric units are distributed along the polymeric chain (figure 1.1). Statistical or random copolymers are those where the different monomers are randomly distributed along the polymer chains. Alternating copolymers have different monomers alternated along the chain. In block copolymers each different monomeric unit form a long segment that are linked together. Graft copolymers have appendages of one type of monomeric units grafted to chains of another one.[1]

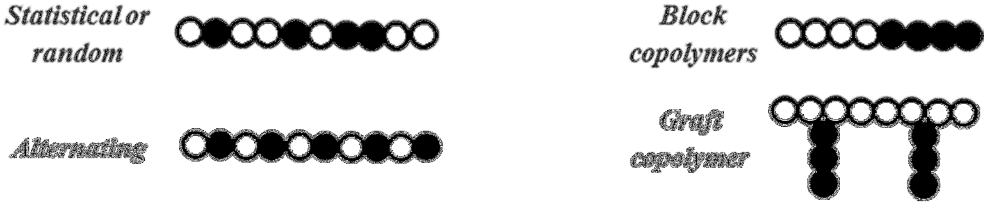


Fig 1.1 Scheme of different types of copolymers with two repeating units.

---

[1] L. Y. Qiu, Y.-H. Bae, *Pharm. Res.,* **2006**. 23, 1-30. *(**doi:** 10.1007/s11095-005-9046-2).*

Among them, block copolymers (BCs) are of particular interest because of their applications in nanotechnology, which are related to the BC behavior in bulk (no solvent) and in solution. If the polymeric segments are immiscible they tend to segregate in the bulk state. However, as the segments are chemically linked, this segregation only occurs at the nanoscale forming well-defined periodic structures. The morphology of the microphase separation depends on the molecular mass of the blocks, their volume fraction and their compatibility. Figure 1.2 illustrates these morphologies.

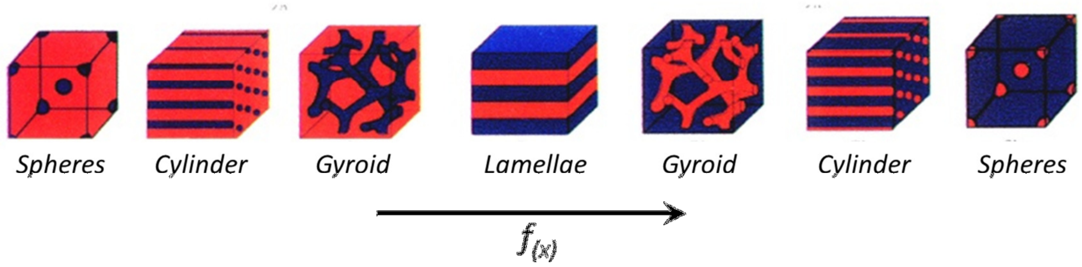


Fig.1.2 Morphologies encountered for BCs (diblock) in bulk as a function of the volume fraction of one of the blocks (f), each color represents a block (adapted from ref. 2)

When a BC is formed by hydrophobic and hydrophilic blocks, the resulting amphiphilic BCs also tend to segregate in selective solvents, in particular water. Thus, the main advantage of amphiphilic BCs is their ability to self-assembly in water, forming organized structures having at least one dimension at the nanoscale. Figure 1.3 illustrates some of these self-assembled structures.

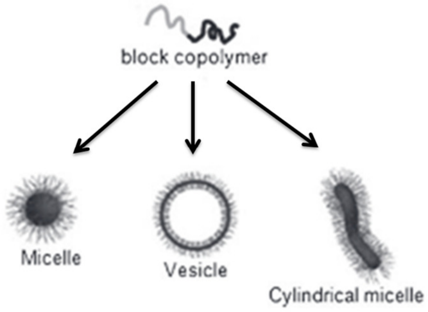


Fig.1.3. Examples of self-assembled structures formed by amphiphilic BCs in solution (adapted from ref.3)

Micelles and vesicles arise from interactions between the dispersion medium and the amphiphilic molecules. The main difference among them is the hollow cavity present in vesicles which comes from a spherical double-wall formation or membrane

---

[2] S. Mahajan, S. Renker, P. F. W. Simon, J. S. Gutmann, A. Jain, S. M. Gruner, L. J. Fetters, G. W. Coates, U. Wiesner. *Macromol. Chem. Phys.* **2003**, 204, 1047–1055. *(**doi**.: 022-1352/2003/0805–1047$17.50p.50)*

[3] J.-W. Park, H. Kim, M. Han. *Chem.Soc.Rev.*, **2010**, 39, 2935–2947. *(**doi:** 10.1039/b918135k).*

(hydrophilic-hydrophobic-hydrophilic) resulting from the aggregation of BCs. Micelles can mainly be spherical or cylindrical, having a hydrophobic internal core and a hydrophilic outer layer or shell, thus maximizing favorable interactions.

Polymeric micelles and vesicles (also known as polymersomes), which have a higher stability than conventional low molecular weight amphiphilic, can be employed as nanocarriers for drug delivery. In fact, many drugs have a limited action because their hydrophobicity influences the optimum absorption and bioactivity of the drug.[4] To minimize this problem, polymeric micelles and vesicles can be used to encapsulate drugs, increasing the level of the drug in aqueous media.[5] Polymeric vesicles are even more interesting because of they can transport hydrophobic and hydrophilic molecules, either in the membrane or in the hollow internal cavity respectively.

Apart from stabilizing and carrying cargo molecules, drug delivery using polymeric nanocarriers might also be improved by the response to specific stimuli. The design of BCs whose properties change in response to changes on the environment imparts to nanocarriers of response to stimuli such as pH, temperature or light. Because conventional delivery systems have several shortcomings regarding the efficiency and specificity of dosage,[6] stimuli responsive BCs micelles and vesicles systems have emerged as a viable option that provides therapeutic efficacy advantages for a progressive and on demand release.[7,8]

1.2 Supramolecular Polymers

Supramolecular chemistry is defined as the chemistry of molecular arrangements and intermolecular bonds. The focus of supramolecular chemistry is the complex interactions and association resulting from two or more chemical species held together by non-covalent forces.[9] Intermolecular forces that lead to formation of supramolecular structures can be secondary bonds, hydrogen bonds, donor-acceptor interactions (type Lewis acid-base) or ionic interactions. Such forces are important in the formation of

---

[4] I. Pepić, J. Lovrić, J. Filipović-Grčić. *Eur. J. Pharm. Sci.,* **2013**, 50, 42-55. *(**doi:**10.1016/j.ejps.2013.04.012.)*

[5] F. Aqil, R. Munagala, J, Jeyabalan, M.V. Vadhanam, *Cancer Lett.* **2013,** 334, 133-141. *(**doi:**10.1016/j.canlet.2013.02.032)*

[6] K. C. Gupta, M. N. V. R. Kumar, Biomaterials, **2000**, 21, 1115-1119. *(**doi:** 10.1016/S0142-9612(99)00263-X)*

[7] G. Gaucher, M.-H. Dufresne, V. P. Sant, N. Kang, D. Maysinger, J.-C. Leroux, *J. Control. Release*, **2005,** 109, 169-188. *(**doi:** 10.1016/j.jconrel.2005.09.034)*

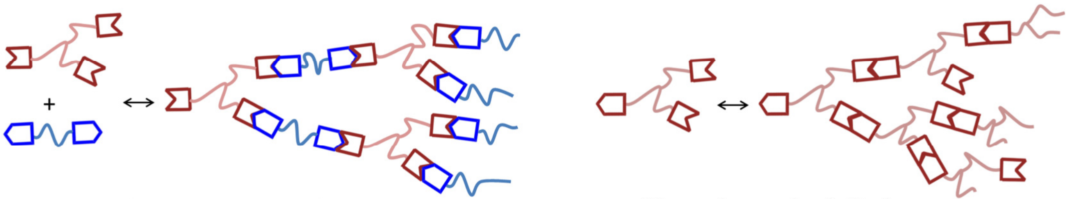
[8] K. Letchford, H. Burt, *Eur. J. Pharm. Biopharm.*, **2007,** 65, 259-269. *(**doi:** 10.1016/j.ejpb.2006.11.009)*

[9] J.-M. Lehn*, Prog. Polym. Sci.,* **2005,** 30**,** 814–831. *(**doi:**10.1016/j.progpolymsci.2005.06.00)*

guest-host complexes, on aggregation states control and they are an important fundamental to crystal engineering. The formation of supramolecular structures is a spontaneous process that happens in a single stage process known as molecular recognition.[10]

The principles of Supramolecular Chemistry can be applied to the synthesis of polymers, which then are known as supramolecular polymers. These polymeric systems are extendable beyond the molecule and use noncovalent interactions to define its assembly and behavior.[11] There are a variety of ways to binding polymers in a supramolecular level, where the supramolecular interactions occurs between complementary groups that allow molecular recognition to form the final polymeric structure (Figure 1.4). In a similar way to what happens in covalent polymer chemistry, in supramolecular polymer chemistry it is also possible to forecast the formation of polymers and copolymers, the reticulation (network formation) or the preparation of any kind of polymeric structure.

Linear Polymer ***Linear Polymer***



Polymeric Network ***Hyperbranched Polymer***

Fig.1.4. Examples of supramolecular polymers.

Among the different type of polymeric structures, side-chain polymers are characterized by functional side groups linked to the polymeric chain and regularly spaced. Side-chain supramolecular polymers can be prepared starting from a preformed polymeric chain modified with functional groups, as pendant or side groups, using different non-covalent interactions such as hydrogen bonding, electrostatic interaction or coordination with metals. The control of these non-covalent interactions is an

---

[10] J.-M. Lehn, *Top Curr Chem*, **2012,** 322, 1–32. *(**doi:** 10.1007/128_2011_256)*
[11] J. D. Fox, S. J. Rowan, *Macromolecules,* **2009**, *42*, 6823-6835. *(**doi:** 10.1021/ma901144t)*

important key to identify and orchestrate the self-assembly of supramolecular entities to prepare this kind of materials. [12]

1.3 Stimuli Responsive Polymers

Stimuli-sensitive polymers are polymeric systems that change their properties in response to small changes in their environment.[13] Particularly, light-responsive polymers are of interest because the change, which is promoted by light, can be very precisely controlled in time and space. In these polymers, the photoresponse is associated with the presence of functional groups which are able to isomerize or react upon irradiation with light of a particular wavelength. Amongst them, the most studied photochromic molecules are the derivatives of azobenzene, which is a simple organic molecule consisting of two aromatic rings linked by an azo bond (N=N). The term "azobenzene" is often used to different molecules with a resembling structure, having an azobenzene core and different substituents.[14]

Due to the presence of N=N bond, azobenzene compounds exhibit *trans-cis* or *E-Z* isomerism. In the *cis* isomer, the phenyl radicals are located at the same side of the N=N bond and in the *trans* isomer, they are at opposite sides. In general, *trans* isomer is thermodynamically more stable than the *cis* isomer. The UV-vis spectrum of the azobenzene shows two characteristic bands, one of higher intensity in the UV region corresponding to a π-π^* transition, and another corresponding to an n-π^* transition in the visible region. Irradiation of an azocompound in the UV region (*i.e.* at the maximum of absorbance of *trans* azobenzene) produces the photoisomerization of the *trans* to the *cis* isomer that is accompanied by a change in the geometric shape and the polarity of the molecule.[15] This isomerization is a reversible process and it is possible to return to the *trans* isomer either by using visible light radiation (*i.e.* irradiating at the n-π^* band of the *cis* azobenzene) or thermally. This process is schematically represented in Figure 1.5

---

[12] M. J. Krische, J.-M. Lehn, E.cheung, G. Vaughn, A. L. Krische, *C. R. Acad. Sci. Paris*, **1999,** 2, 549-556. ***(doi.:** 1387-1609/99/00020549)*

[13] A. W. Bosman, R. P. Sijbesma, E. W. Meijer, *Materials Today*, **2004**, 7, 34-39. ***(doi:** 10.1016/S1369-7021(04)00187-7)*

[14] A. Castelón, *Final Master Project*, Science Faculty, University of Zaragoza, June **2014**.

[15] R. Klajn, *Pure Appl. Chem*, **2010**, 82, 2247–2279. ***(doi:**10.1351/PAC-CON-10-09-04)*

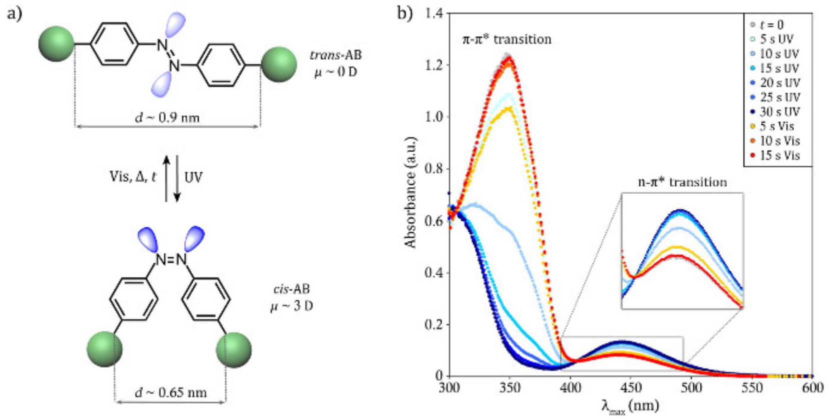


Fig 1.5 a) Representation of the trans-cis azobenzene isomerization and b) evolution of the UV-vis spectrum induced by illumination with UV or visible light. (Ref.:15)

The incorporation of these units in amphiphilic block copolymers makes them of interest in the preparation of nanocarriers with a programmed response to light, used as stimulus for the delivery of cargo molecules.

1.4 Antecedents of the Final Master Project

1.4.1 Supramolecular Copolymers with Azobenzene

As it has been mentioned above, micellar self-assemblies obtained from amphiphilic BCs have been widely studied because of their potential as drug delivery systems. The present project has been performed in the *'Liquid Crystal and Polymer (CLIP) group'* of the University of Zaragoza and is connected with previous work done in the group within the field of amphiphilic BCs. The first examples described by this group were amphiphilic linear-dendritic BCs functionalized with 4-cyanoazobenzene units, for which the formation of cylindrical, sheet-like, tubular micelles and polymer vesicles were described. In particular, vesicles showed response to UV light irradiation suffering deformation or collapsing.[16]

Nevertheless, changing the 4-cyanoazobenzene moiety by the 4-isobutyloxyazobenzene one, it was possible to obtain vesicles with a highly improved response to light. The reason lays in the higher polarity difference between *cis* and *trans* isomers and the lower interpenetration of these azobenzene moieties, thus facilitating

---

[16] J. del Barrio, L. Oriol, C. Sánchez, J. L. Serrano, A. Di Cicco, P. Keller, M. H. Li, *J. Am. Chem. Soc.*, **2010**, 132, 3762-3769. ***(doi:** 10.1021/ja9083946)*

the collapsing/opening of self-assemblies under radiation with a low intensity UV lamp. The study was complemented with the encapsulation of a fluorescent probe to follow the controlled release of the cargo molecules under radiation.[17]

Supramolecular photoresponsive BCs are other topic of interest in this group. Recently, the group described photoresponsive poly(methyl methacrylate)-*b*-poly(4-vinylpyridine) where azobenzene groups are linked to the pyridine moieties by H-bonding.[18] These photoswitchable supramolecular BCs responded to light and the azobenzene orientation can be induced by irradiation with a linearly polarized light.[19] Also, they have been described supramolecular BCs with azo moieties linked by multiple hydrogen bonds interactions, using in this case 2,6-diacylaminopyridine moities repeating units and derivatives of azobenzenes having thymine or carboxyl-terminal groups.[20]

1.4.2 Preparation of Self-assemblies from Amphiphilic Block Copolymers

Polymer nanoparticles can be generated either during the polymerization of the monomers or later on from a dispersion of the polymer. Different methods are accessible that can be selected depending on the desired properties such as particle size, particle distribution, use of additives or surfactants, and/or the application devised for the nanoparticles, amongst others.[21]

Focusing attention on the preparation of aqueous dispersions of nanoparticulated self-assemblies from a preformed amphiphilic BC, the method has not only impact on the size and size distribution of the self-assemblies but also on their stability. In order to self-assemble, a BC must be present in sufficient quantity in the solution. The specific concentration required for micelle formation is referred to as the critical micelle concentration (CMC). Below the CMC, only free, randomly distributed single chains are present, but above the CMC, both micellar self-assemblies and single chains co-

---

[17] E. Blasco, J. del Barrio, C. Sánchez-Somolinos, M. Piñol, L. Oriol, *Polym. Chem.,* **2013**, 4, 2246-2254. *(**doi**.:dx.doi.org/10.1021/ma4009725)*

[18] J. del Barrio, E. Blasco, L. Oriol, R. Alcalá, C. Sánchez-Somolinos, *J. Polym. Sci. Part A: Polym. Chem.* **2013**, 51, 1716–1725. *(**doi:** 10.1002/pola.23920)*

[19] J. del Barrio, E. Blasco, C. Toprakcioglu, A. Koutsioubas, O. A. Scherman, L. Oriol, C. Sánchez-Somolinos, *Macromolecules*, **2014**, 47, 897–906. *(**doi:** dx.doi.org/10.1021/ma402369p)*

[20] A. Concellón, E. Blasco, M. Piñol, L. Oriol, I. Díez, C. Berges, C. Sánchez-Somolinos, R. Alcalá, *J. Polym. Sci. Part A: Polym. Chem.,* **2014,** 52, 3173-3184. *(DOI: 10.1002/pola.27373*

[21] J. P. Rao, K. E. Geckeler, *Prog. Polym. Sci.*, **2010,** 36, 887-913. *(**doi:**10.1016/j.progpolymsci.2011.01.001)*

exist.[22] Amphiphilic copolymers with a low hydrophobic content can be directly dispersed in water.[23] However, whenever the water solubility is too low, methods can be used that require the temporary use of a ‘good’ organic solvent of the polymer. In this case, selection of the organic solvent, techniques to efficiently mixing of the organic and water phases and removal of the organic solvent are some of the critical issues to take into consideration.

If a water immiscible volatile solvent (dichloromethane, chloroform or ethyl acetate are common examples) is used, aqueous dispersions of the polymer self-assemblies in water can be obtained from an emulsion prepared by high-speed mixing or ultrasonication after evaporation of the solvent either by continuous magnetic stirring at room temperature or under reduced pressure. This can be a simple method but possible coalescence during the evaporation process of the generated nanodroplets may affect the final particle size and morphology. This ‘solvent evaporation method’ has also been used with water miscible solvents such as acetone or THF.[21, 22]

The ‘solvent displacement’ method or ‘nanoprecipitation’ is an alternative to obtain polymer self-assemblies. In this case, a good organic solvent, which is miscible with water and easy to remove by evaporation, is used. The organic solution of polymer can just be slowly dropped in water under moderate stirring, and the organic solvent removed through evaporation.[24,25] Nanoparticles with a well-defined size characterized by a narrow distribution are formed instantaneously during the rapid diffusion of the polymer solution in the aqueous phase. Reversing this order by adding the aqueous phase to the organic phase also leads to the formation of nanoparticles.

Dialysis of an organic solution against water or water/organic solvent mixtures of increasing water content is another simple alternative. Polymer is dissolved in an organic solvent and placed inside a dialysis tube with proper molecular weight cutoff [21]. Like in the nanoprecipitation method, the displacement of the solvent inside the membrane is followed by the progressive aggregation of polymer due to a loss of solubility and the formation of homogeneous suspensions of nanoparticles.

---

[22] Z. L. Tyrrella, Y. Shena, M. Radosz, *Prog. Polym. Sci.*, **2010,** 35, 1128-1143.*(**doi:** 10.1016/j.progpolymsci.2010.06.003)*

[23] S. Tanodekaew, R. Pannu, F. Heatley, D. Attwood, C. Booth, *Macromol. Chem. Phys.*, **1997,** 198, 927-944. .*(**doi:** 10.1002/macp.1997.021980402)*

[24] S. Galindo-Rodriguez, E. Allemann, H. Fessi, E. Doelker, *Pharm. Res.*, **2004,** 21, 1428–1439. *(**doi:** 10.1023/B:PHAM.0000036917.75634.be)*

[25] J. Ford, P. Chambon, J. North, F. L. Hatton, M. Giardiello, A. Owen, S.P. Rannard, *Macromolecules* **2015**, 48, 1883−1893.*(**doi:** 10.1021/acs.macromol.5b00099).*

Nevertheless, preparation of stable and uniform self-assemblies dispersions is not an obvious task that has much of trial-error and multiple variations and combinations of each technique can be found in the literature. Quaglia and co-workers prepared self-assemblies by the melting-sonication procedure which avoids the use of toxic solvents. The BC is melted in water by heating above the copolymer melting temperature and sonicated using ultrasonic probe. The authors demonstrate that the strategy is efficient for the generation of nanoparticles that also have a larger hydrodynamic diameter size than those obtained from dialysis.[26]

Jérôme and coworkers compared three methods with amphiphilic poly(ethylene oxide)-*b*-poly(ε-caprolactone) and poly(ethylene oxide)-b-poly(-methyl-ε-caprolactone) They concluded that direct dialysis of organic solutions against water does not allow an acceptable size control of the self-assemblies. However, rapid addition of the organic solution in water or water in the organic solution, prior to dialysis, allow well-defined particles to be formed. The addition of some water to the organic phase appears to 'freeze' the micelles, leading to a more reproducible size profile. Also, there is evidence that the choice of organic solvent (DMSO, DMF, DMAc, THF) influences the size of the micelles.[27]

Therefore, addition of water to the organic solution until aggregation occurs, also referred as the co-solvent method, has been recurrently used in the literature and it has been the method used by the *CLIP group* of the University of Zaragoza. [16,17,18],[28] In general, this method has been carried out by previous solution of the BC in THF (water-miscible organic solvent in which both blocks are soluble) and slowly addition of water till aggregation followed by dialysis.

---

[26] F. Quaglia, L. Ostacolo, G. De Rosa, M. Immacolata, L. Rotonda, M. Ammendola, G. Nese, G. Maglio, R. Palumbo, C. Vauthier, Intern. J. Pharmaceutics, **2006,** 324, 56–66 *(**doi:**10.1016/j.ijpharm.2006.07.020)*

[27] P. Vangeyte, S. Gautier, R. Jérome. *Colloids and Surfaces A: Physicochem. Eng. Aspects*, **2004,** 242, 203–211 *(**doi:**10.1016/j.colsurfa.2004.04.070)*

[28] E. S. Leea, Y. T. Ohb, Y. S. Younc, M. Namd, B. Parkd, J. Yund, J. H. Kimd, H.-T. Songb, K. T. Oh, *Colloids and Surfaces B: Biointerfaces*, **2011**, 82, 190–195. *(**doi:**10.1016/j.colsurfb.2010.08.033)*

2. OBJECTIVE

The aim of the project is to gain knowledge on the self-assembly of amphiphilic linear-linear BCs that can be functionalized in a versatile and simple manner by supramolecular chemistry incorporating moieties to endow with systems having a controlled response to changes on the environment. According to this general goal, the project proposes the synthesis and characterization of an amphiphilic BC containing 2,6-diacylaminopyridine units, the study of the self-assembly process in aqueous solutions, as well as the supramolecular functionalization with azobenzene moieties. The presence of azobenzene moieties should provide light response as well as the presence of 2,6-diacylaminopyridine (DAP) moieties by themselves might also provide pH response. Because 2,6-diacylaminopyridine can form multiple hydrogen bonds (molecular recognition) with thymine, by using thymine-containing azobenzene units it should be possible to generate light sensitive nanoparticles that can be used for the on demand release of cargo molecules.

2.1 Synthesis and Characterization of the Amphiphilic Block Copolymer

The first task of this project is the synthesis of the amphiphilic block copolymer, coded as PEG-*b*-PDAP, which has polyethylenglycol having a molar mass around 10000 as the hydrophilic block, and a hydrophobic block derived from a 2,6-diacylaminopyridine (DAP) methacrylic monomer (Figure 2.1). To have a good control on the architecture, PEG-*b*-PDAP is prepared by a controlled radical polymerization process, in particular by RAFT polymerization.

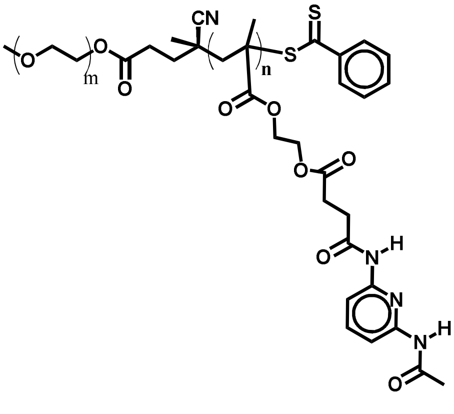


Fig. 2.1. Chemical structure of PEG-b-PDAP

2.2 Synthesis and Characterization of Supramolecular Amphiphilic Block Copolymers

Supramolecular copolymers are prepared by interactions of the DAP repeating units with an azocompound having thymine groups by means of hydrogen bonds; this interaction happens in according to the scheme in Figure 2.2, wherein x% indicates the percentage of DAP units complexed with thymine by hydrogen bonging. Thymine binds complementary 2,6-diacylaminopyridine moieties by three hydrogen bonds. Thymine-containing 4-isobutyloxyazobenzene (Figure 2.3) (coded as tAZO) was used, which was available in the research group.

Previously in the *CLIP group* has been performed supramolecular polymer where each DAP units was "occupied" by a tAZO units. In order to verify the effect of free DAP units has on the final characteristics of supramolecular polymer (coded as PEG-b-PDAP·tAZO x%), here, is proposed different x% (25%, 50%, and 75%).

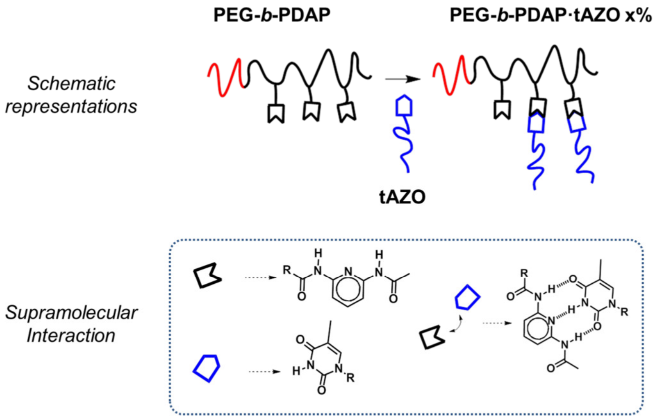


Fig 2.2 . Schematic representation for the formation of the supramolecular polymers.

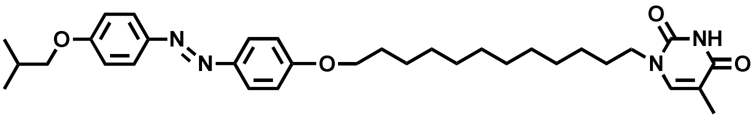


Fig. 2.3. Molecular scheme of tAZO

2.3 Preparation and Characterization of Polymeric Nanoparticles

The ultimate task of the project is the preparation of polymeric nanoparticles from PEG-*b*-PDAP and PEG-*b*-PDA·tAZO x% by different methodologies: co-solvent method and ultrasonic dispersion, using different experimental conditions. The study the morphology of polymeric nanoparticles is carried out by transmission electron microscopy.

3. RESULTS AND DISCUSSION

3.1 Polymerization by Reversible Addition-Fragmentation Chain Transfer (RAFT) and Characterization of the Block Copolymer

The synthesis of the aimed amphiphilic BC (PEG-*b*-PDAP) required a controlled radical polymerization method in order to have a macromolecule with a well-defined structure. For this purpose, Reversible Addition-Fragmentation Chain Transfer (RAFT) was selected. This polymerization method was first described by the CSIRO group and it was based on the use of a chain transfer agent (CTA).[29] The mechanism of this polymerization is shown in Figure 3.1 and first requires the use of a conventional radical initiator. The presence of the CTA gives rise to an addition-fragmentation equilibrium and a subsequent minimization of the termination process. The chemical nature of the CTA depends on the type of monomers to be polymerized.[14] An important issue is that the final polymer is isolated as a stable material with the majority of the macromolecules having the CTA fragment at their end. Therefore, these macromolecules can be used as chain transfer agents (also known as macro-CTA due to its polymeric nature) in a subsequent polymerization process with other monomer to obtain a BC.

---

[29] G. Moad, E. Rizzardo, S. H. Thang, *Aust. J. Chem.*, **2005**, 58, 379-410 ***(doi:** 10.1071/CH05072)*

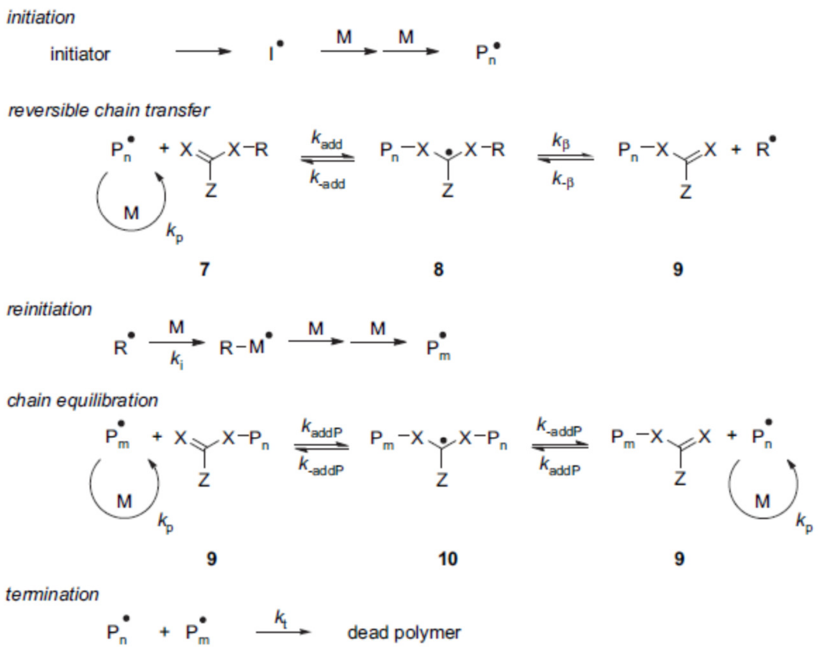


Fig.3.1. Mechanism of RAFT polymerization. (Ref.29)

In this project, a commercial macro-CTA based on polyethyleneglycol was used to polymerize the 2,6-diacylaminopyridine methacrylate (Figure 3.2). According to the supplier, the macro-CTA had a molecular mass of 10000 (approx. m=227), value that was also checked by mass spectrometry before being used. The polymerization was carried under Argon (inert atmosphere) at 80ºC for 5h using freshly distilled *N,N*-dimethylformamide (DMF) as solvent. After polymerization PEG-*b*-PDAP was precipitated into cold diethyl ether and isolated by vacuum filtration.

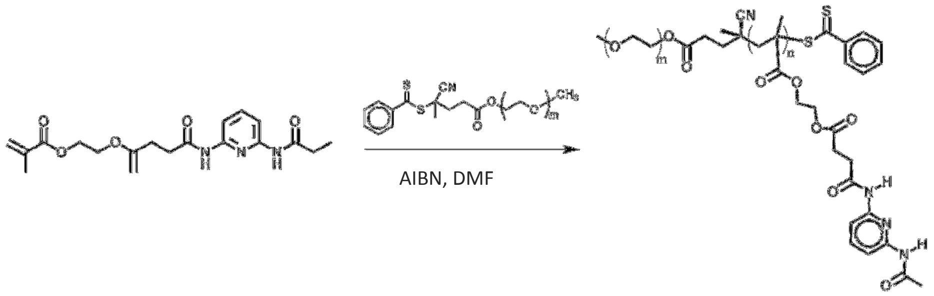


Fig 3.2. Synthesis of PEG-b-PDAP by RAFT polymerization.

Chemical structure of PEG-*b*-PDAP was corroborated by FTIR and ^{1}H RMN (see Appendix A.1). The relative average molecular mass and molecular mass distribution (or dispersity) were determined by gel permeation chromatography (GPC) using an evaporative light scattering detector and poly(methyl methacrylate)s as standards. Average molar masses (relative to PMMA as standards) obtained from GPC for PEG-*b*-PDAP copolymer were M_m = 14900 g/mol, M_n = 13400 g/mol, and dispersity M_m/M_n = 1.11. Composition of the copolymer was determined ^{1}H RMN. Taking the integration of the peaks corresponding the methylene groups of the PEG block as a reference (and considering a degree of polymerization of m=227, according to commercial value checked by mass spectrometry), it was possible to calculate the relative integration of peaks corresponding to the DAP repeating units (e.g. by considering the pyridine and amide protons of this units). The calculated degree of polymerization of the PDAP block (n) by this method was around 28 that was used as value of reference for the subsequent functionalization by hydrogen bonding (according to that value M_n is around 20000).

The thermal stability of PEG-*b*-PDAP was studied by thermogravimetric analysis (TGA), wherein it was possible to observe that the polymer had an adequate stability up to 200°C, and had no significant mass loss associated to volatile compounds (see Appendix A.2). Differential Scanning Calorimetry (DSC) (See Section E.1 in Annexes) revealed that PEG-*b*-PDAP is an amorphous material having a single glass transition temperature (Tg) at -3ºC as it is observed in Figure 3.3. The presence of only one Tg is a very significant result as BCs usually exhibits the thermal transition of all blocks due to their immiscibility. It should be remarked that the PEG separated block is semicrystalline (it has a very low Tg and a Tm around 50ºC) and the homopolymer PDAP (the other block) previously synthesized in the labs of the *CLIP group* is an amorphous material with a Tg at 73ºC. Consequently the new Tg at -3ºC corresponds to this miscible system.

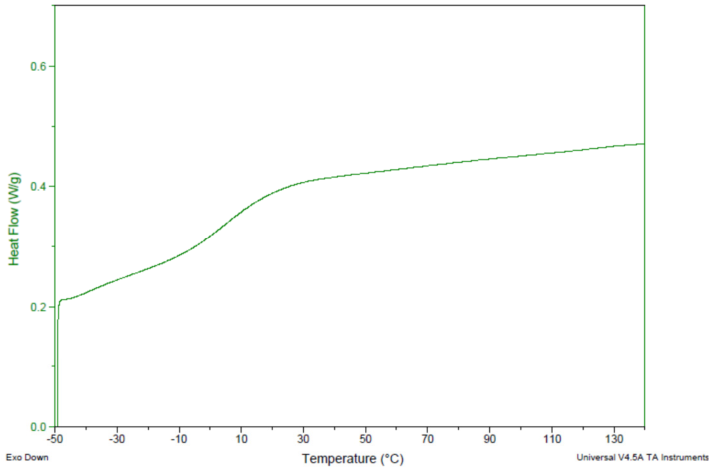


Fig. 3.3. DSC curve of PEG-b-PDAP registered on the second heating scan at 10 ºC/min

3.2 Preparation and Characterization of Supramolecular Block Copolymers

The purified PEG-*b*-PDAP was used for the preparation of micelles (see section 3.3 and 3.4) but also for the further preparation of supramolecular BCs using the thymine containing 4-isobutyloxyazobenzene (tAZO) that is capable of forming three hydrogen bonds with the 2,6-diacylaminopyridine unit (Figure 2.3 and 2.4).

Several supramolecular copolymers with variable content of tAZO were prepared. The fully complexed copolymer (100% of functionalized 2.6-diacylaminopyridine units, *i.e.* 1:1 stoichiometry AZO:DAP) was previously studied in the *CLIP group.*[14] In this project, only 75%, 50%, and 25% of the DAP units were complexed to tAZO in an attempt to decrease the percentage of hydrophobic photochromic part and studied its influence on the micellization process and stability of the final micelles.

For the preparation of the supramolecular BCs, both components (*i.e.* the azocompound and the BC) were weighted, dissolved in THF and finally the solvent was slowly evaporated (under continuous gentle mechanical stirring) at room temperature. The polymers were thus obtained by supramolecular recognition by hydrogen bonding. These supramolecular BCs were analyzed by ^{1}H RMN and FTIR spectroscopies and TGA, DSC and optical microscopy. FTIR and ^{1}H-RMN were relevant techniques to determine hydrogen bonding associations. DSC and optical microscopy were useful to determine thermal transitions and morphology, and TGA was done to analyze the thermal stability. The results corresponding to the precursor PEG-*b*-PDAP and to the supramolecular resulting BC PEG-*b*-PDAP·tAZO x% (where x= 75, 50, 25, which corresponds to the percentage of complexation of DAP) were compared. FTIR spectra were registered using KBr pellets and are shown in Appendix A.3. Formation of hydrogen bonding was better demonstrated by ^{1}H NMR as can be seen in Figure 3.4. In fact, signals corresponding to the protons involved in hydrogen bonding were shifted in the complexed supramolecular BC with respect to the starting materials. Protons coded as A,A’ of the amide group of DAP and proton B of the thymine ring were shifted to higher chemical shifts due to supramolecular assembly.

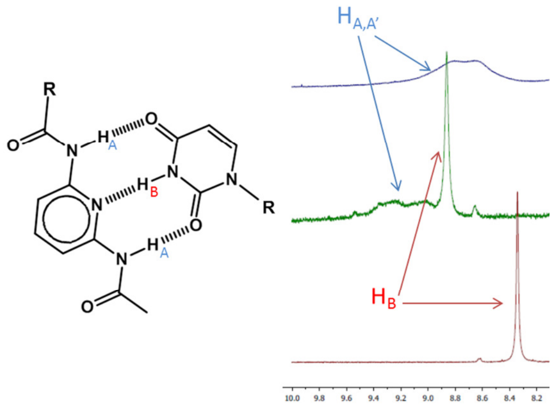


Fig. 3.4. ^{1}H RMN (CDCl$_3$) of PEG-b-PDAP (blue), PEG-b-PDAP • tAZO 50% (green) and tAZO (red).

Thermal stability associated to mass loss of PEG-*b*-PDAP·tAZO x% supramolecular copolymers, was studied by TGA and the thermal transitions by DSC. Most relevant data are collected in Table 3.1. BCs show an adequate stability up to 200 ºC similar to the starting BC. TGA results are available in appendix A.4.

Table 3.1. Thermal parameters obtained by TGA and DSC (second heating scan, 10ºC/min).

Polymer	**T loss 5% wt (1)**	**T onset DGTA (2)**	**T Max DGTA (3)**	**Tg DSC (4)**	**Tm$_1$/Tm$_2$ DSC (5)**
PEG-*b*-PDAP	249 ºC	227 ºC	250 ºC	-3	-
PEG-*b*-PDAP·tAZO 75%	259 ºC	230 ºC	253 ºC	42	50/156
PEG-*b*-PDAP·tAZO 50%	249 ºC	224 ºC	269 ºC	-12 / 42	49/147
PEG-*b*-PDAP·tAZO 25%	249 ºC	227 ºC	227 ºC	-8	-

(1) Temperature associated to 5% mass loss detected in the thermogravimetric curve; (2) Onset of the decomposition peak (first derivative); (3) Maximum temperature of the decomposition peak (first derivative); (4) Glass transition temperature; and, (5) Melting temperature.

As it was mentioned in section 3.1.2, PEG-*b*-PDAP is an amorphous material with Tg at -3ºC. In previous work of the *CLIP Group,*[14] thermal behavior of the tAZO, fully complexed supramolecular BC, PEG-*b*-PDAP·tAZO 100%, and the corresponding supramolecular homopolymer PDAP·tAZO (taken for comparative purposes) were investigated (tAZO is a crystalline material which melts at Tm 166 ºC). The complexed homopolymer PDAP·tAZO displays a glass transition at around 35ºC followed by a cold crystallization and subsequent melting of the crystalline complexed PDAP block at

148ºC. On the DSC heating curve of the fully-complexed supramolecular BC, PEG-*b*-PDAP·tAZO 100% it was observed a Tg at 38ºC (similar to the homopolymer PDAP·tAZO) followed by a melting peak at 48ºC (overlapped with Tg) corresponding to the PEG block. After this peak, it was observed a cold crystallization and a subsequent melting peak at 148 ºC corresponding to the PDAP·tAZO block. The appearance of several thermal events ascribable to the different blocks (PEG and PDAP·tAZO) suggested that complexation induced microphase separation by decreasing the miscibility between blocks.

In the case of PEG-*b*-PDAP·tAZO 75%, the DSC was very similar to the previously described for the fully complexed. A Tg at 42ºC with an overlapped melting peak at 50ºC (melting of PEG block) followed by a cold crystallization at the melting peak at 156ºC (Figure 3.5a) were observed. In the case of PEG-*b*-PDAP·tAZO 50%, the DSC curve was more complicated, although it can be considered as the results of two contributions: on one hand the complexed block copolymer and on the other hand, the non-complexed block copolymer. Thus, a first Tg around -12ºC was detected and may correspond to the non-complexed and miscible BC (slightly shifted with respect to PEG-*b*-PDAP detected at -3ºC) and then a Tg at 42ºC and two melting peaks at 49 and 147ºC similar to the previously observed in the 75% complexed. These peaks have lower enthalpy contents due to the low percentage of complexation.

Finally, the DSC of PEG-*b*-PDAP•tAZO$_i$ 25% was more similar to PEG-*b*-PDAP (non complexed), due to the low content of complexation and only a Tg at -8ºC is hardly detected. These results confirm that the complexation of the DAP units favor the segregation of both blocks that are initially miscible in the starting BC.

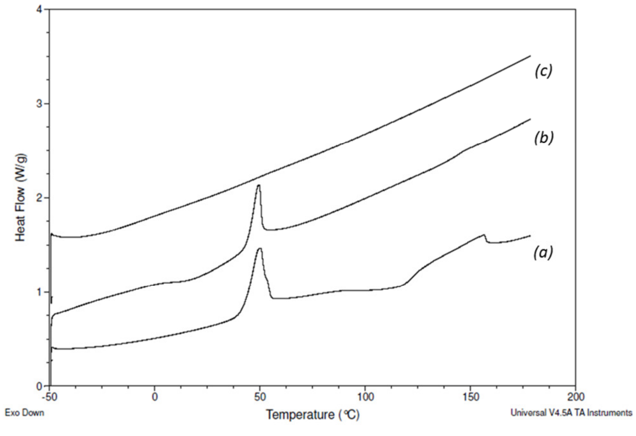


Fig 3.5. DSC curves corresponding to the second heating scans 10ºC/min (previously heated to isotropic state at 200ºC and cooled to -50ºC at 10ºC/min) of (a) PEG-b-PDAP·tAZO 75%; (b) PEG-b-PDAP·tAZO 50%; and (c) PEG-b-PDAP·tAZO 25%

3.3 Preparation and characterization of polymeric nanoparticles: Co-solvent and dialysis methodology

As mentioned above, several methodologies are available for preparing polymeric micelles and vesicles from amphiphilic BCs. In this project, the conventional co-solvent method was first tried. The method has been widely used for many research groups because it affords size homogeneous self-assemblies. Nevertheless, as it will be discussed below, other methodologies have also been investigated.

Co-solvent method consists in the use of two miscible solvents, with distinct polarity and affinity for each block. Based on previous experiences, the initial experiments were carried out using THF as the common solvent for both blocks and water as the selective solvent. In order to obtain self-assembled polymeric nanoparticles, the BC was completely solubilized in THF and then Milli-Q water was slowly added. The self-assembly process was controlled by measuring the solution absorbance at a specific wavelength in a UV-vis spectrophotometer (λ= 650nm in this case). Actually, this procedure, known as turbidimetry, is indeed an indirect measurement of the scattering effect due to the dispersed self-assembled macromolecules. The slow addition of water promotes the segregation of the blocks and formation of micellar assemblies (more energetically favorable), the solution becomes cloudy and disperses more light (apparent increase of absorption). Figure 3.6 represents the data acquisition during a typical experiment using THF/water as solvents. The abrupt change on turbidity indicates micellization. Once formed, the organic solvent is finally removed by dialysis against water.

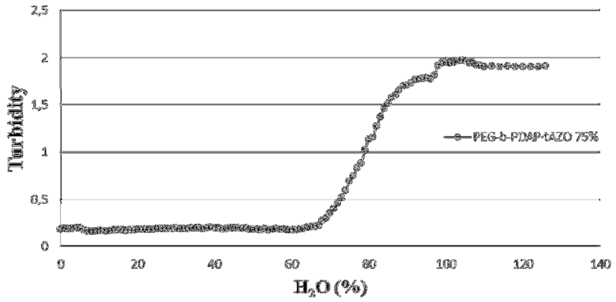
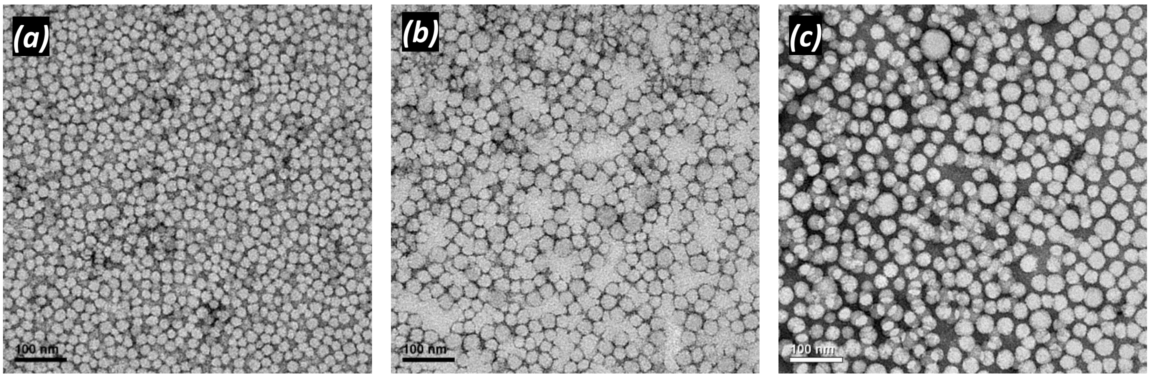


Fig. 3.6. Turbidity change of PEG-b-PDAP·tAZO 75% in THF

When using the system THF/water, spherical and homogenous micelles were generated from PEG-*b*-PDAP as was observed by TEM, see Figure 3.7 *(a)*. A solution with 5 mg of PEG-*b*-PDAP in 1 mL of THF was initially prepared, and small portions (10 µL) of water were slowly added. The total volume of water at the end of the addition was 1.3 mL and all the content was transferred to a dialysis membrane (MWCO: 1000). The dialysis was maintained during 3 days and the final volume registered was 2.5 mL. The size of theses aggregates was measured by TEM and DLS giving average diameters of 26 nm and 38 nm, respectively.

The TEM average diameter was obtained from a collection of data from several TEM images (appendix A5 - Table A.5.1). The intensity size distribution determined by DLS shows a bimodal distribution. When the number size distribution was analyzed it can be observed that 99% of the total population of the sample corresponds to the smallest aggregates characterized by a peak mean of 38 nm, which is in good agreement with the value measured for the micelles by TEM. The second population is characterized by a peak mean of 340 nm that can be ascribed to micellar aggregates as TEM images do not point out the existence of other kind of aggregates.

However, this system was not efficient to prepare micelles from the supramolecular BC PEG-*b*-PDAP·tAZO 75%, neither from polymers with 50% or 25% of tAZO content. With these supramolecular polymers, during the dialysis process, a solid was formed at the bottom of the dialysis bag. Nevertheless, the supernatant aqueous dispersion was studied by TEM to detect the presence of aggregates and spherical micelles were observed having a diameter of approx. 41 nm (Figure 3.7 *(b)*). A hypothesis to explain the results is that the precipitate was mainly tAZO and consequently micelles are mostly of PEG-*b*-PDAP (non-complexed). This supernatant aqueous dispersion was irradiated with UV-light. As tAZO is a light responsive moiety the presence of these units in the micelles can give rise to the collapse or a morphological modification of aggregates as it has been reported by the CLIP group.[14,17,18,19] However, TEM images of these irradiated materials, exhibited a similar morphology to non-irradiated (Figure 3.7 *(c)*), which lead us to think again that the observed yellow precipitate was mainly the azo chromophore and the BC remains dispersed as self-assembled aggregates.



3.7. TEM images of the aggregates obtained by co-solvent method using THF/water for (a) PEG-b-PDAP, (b) PEG-b-PDAP·tAZO 75%, and (c) UV-light irradiated sample of PEG-b-PDAP·tAZO 75%

The UV-light irradiation process of the supernatant dispersion isolated from the self-assembly of PEG-*b*-PDAP·tAZO 75% was also monitored by UV-vis spectrometry. In fact, a displacement of the maximum of absorbance and an increase of the n-π* band should be expected for the azobenzene included in the micelles upon increasing the time of radiation (Figure 3.8). However only a decrease of absorbance is detected, probably associated to additional precipitation, but it gave no clear evidence of the *trans* to *cis* isomerization.

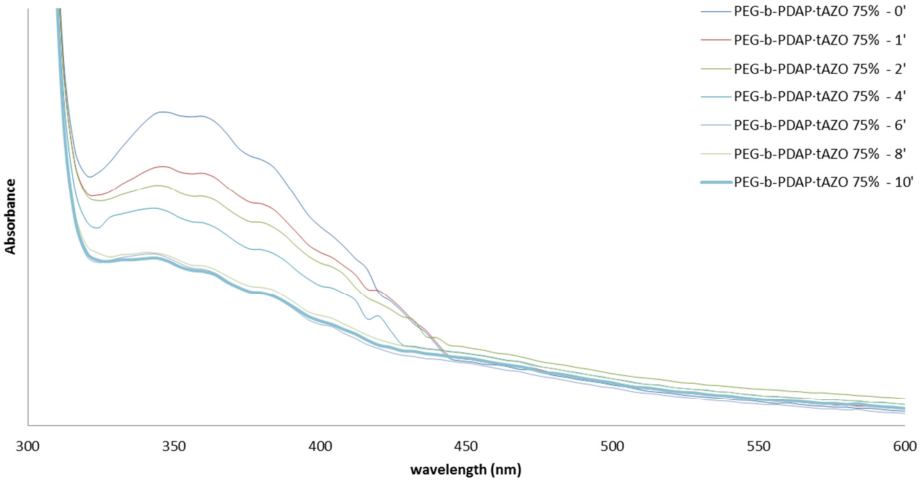


Fig 3.8. UV-vis spectra of the PEG-b-PDAP·tAZO 75% supernatant aqueous dispersion upon UV light irradiation

Similar results were obtained for PEG-*b*-PDAP·tAZO 50% and PEG-*b*-PDAP·tAZO 25% supramolecular BCs. The corresponding TEM images are collected in Figure 3.9.

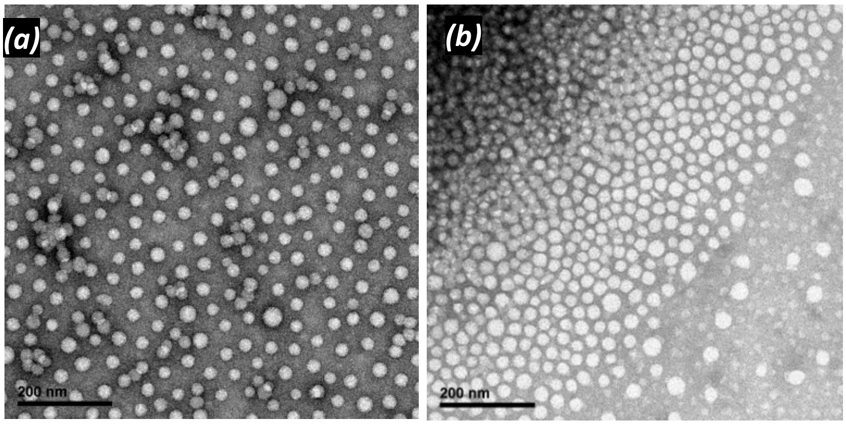


Fig 3.9. TEM images of the aggregates obtained by co-solvent method using THF/water for (a) PEG-b-PDAP·tAZO 50% and, (b) PEG-b-PDAP·tAZO 25%.

In an attempt to avoid the formation of precipitate and prepare stable micelles from the supramolecular BCs, different experimental conditions of the co-solvent method were studied using PEG-b-PDAP·tAZO 75%. As the 2,6-diacylaminopyridine is pH-sensitive, milliQ-water (usually used in the lab of the group for preparing this kind of self-assembled polymeric nanoparticles) was replaced by a phosphate buffer of pH 7.4. However, the results were very similar. Secondly, THF was replaced by other organic solvents, miscible with water, as acetone and dioxane but precipitation was not avoided and the micelles were hardly observed by TEM (see Figure 3.10).

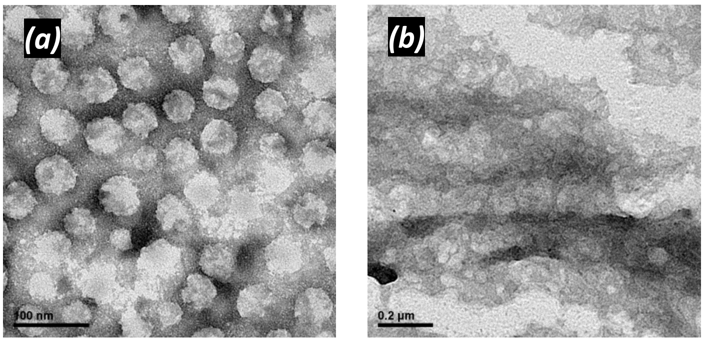


Fig 3.10 TEM Image PEG-b-PDAP·tAZO 75% aggregates obtained by co-solvent self-assembly method using: (a) THF/Buffer: and (b) Dioxane/water.

Due to the encountered problems with supramolecular BCs, efforts were focused on the preparation of polymeric nanoparticles from the starting BC, testing different methodologies. First, an attempt was carried out to avoid the dialysis process and to make easier and more direct the preparation of the self-assemblies. Second, sonication of the BC in water either with or without the aid of an organic solvent was tried (see next section).

To avoid dialysis, THF was replaced by DMSO a water miscible organic solvent of low toxicity.[30,31,32,33] Therefore, water was gradually added over a DMSO solution of PEG-*b*-PDAP of 1 mg/mL. Unfortunately, DMSO/water is not so efficient in comparison with THF/water system due to the presence of precipitate at the end of process (following the same steps than in THF/water). The sample was analyzed by TEM (Figure 3.11 *(a)*) where is possible to see micelles. To avoid precipitation the addition of water was stopped just when solution became turbid. However, at this point, micelles were not properly formed (Figure 3.11 (b)).

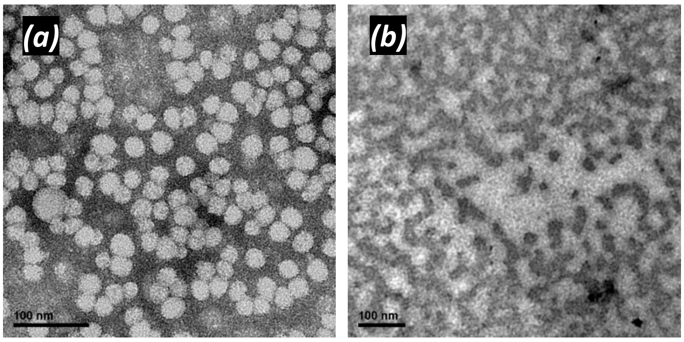


Fig 3.8. TEM images of aggregates obtained from PEG-b-PDAP using DMSO/water.

3.4 Preparation and characterization of polymeric nanoparticles: Sonication

Self-assembly of the BC was also checked by sonication using an ultrasonic processor provided with an adequate sonotrode. Advantages of this technique are the low running time and the simple preparation process that is easily reproducible. In an ultrasonic processor, working conditions are a combination of cycle and amplitude. *Cycle* is the factor for regulating ON/OFF mode of the ultrasonic processor. The value fixed for the cycle is equal to the acoustic irradiation time in seconds and the difference to 1 is the pause time (off); i.e., a cycle value of 1 means that the ultrasonic processor is continuously switched ON; however, a value of 0.6 means that the ultrasonic processor is 0.6 seconds ON and 0.4 seconds OFF. The *amplitude* regulates the ultrasonic output of the sonotrode. To avoid an excessive heating and possible degradation of the sample the maximum time of acoustic irradiation was fixed in intervals of 5 min, for all cases.

In a first experiment PEG-*b*-PDAP·was dissolved in DMSO (1mL) and small portions (10µL) of water were added up to a final volume of 2 mL. After each

---

[30] USP 28–NF23 - United States Pharmacopeial Convention, *Inc., Rockville*, MD, **2005**, 662–664.
[31] European Pharmacopoeia - Council of Europe, *Strasbourg*, **2002**, 1055.
[32] Material Safety Data Sheet – *Sigma Aldrich* - Product Details 39291, **2015.**
[33] British Pharmacopoeia, **1993** edition supplement, The Stationery Office, London, **1996**.

addition, the sample was sonicated for 30s (70 Amp/0.5 cycle) (Figure 3.12 *(a)*). Under these conditions, precipitation was not observed, although this preparation method was modified as it was difficult to follow up the process. Instead, water was added in one portion to the DMSO solution and then sonicated (two intervals of 5 min) (Figure 3.12 *(b)*).

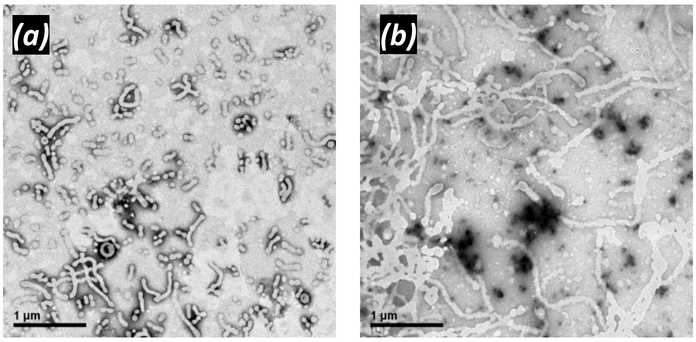


Figure 3.12. TEM images of PEG-b-PDAP aggregates prepared from a sonicated DMSO solution with (a) slow and (b) rapid water addition (see text for experimental conditions).

In a second experiment, dichloromethane (DCM) was used as organic solvent that can be easily removed during the sonication process due to the heating of the solution. PEG-*b*-PDAP (1mg) was first dissolved in DCM (10µL), then water (1mL) was added and finally the system was sonicated (70Amp/0.5cycle) in two intervals of 5 min (total time=10 min). When the resulting dispersion was studied by TEM, it was observed the formation of polymeric spherical nanoparticles together with cylindrical and toroidal micelles that seems to be the previous stage of aggregation before formation of spherical micelles (Figure 3.13 *(a)*). In a subsequent experiment, PEG-*b*-PDAP was directly dispersed in a mixture of DCM (10µL)/water (1mL) (i.e. without previous solubilizing of the polymer) and sonicated under the same conditions giving similar results (Figure 3.13 *(b)*).

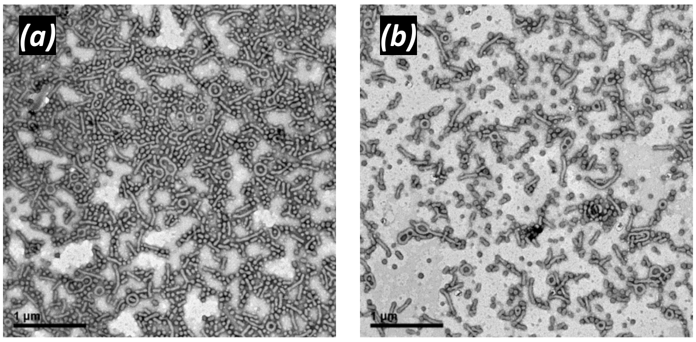
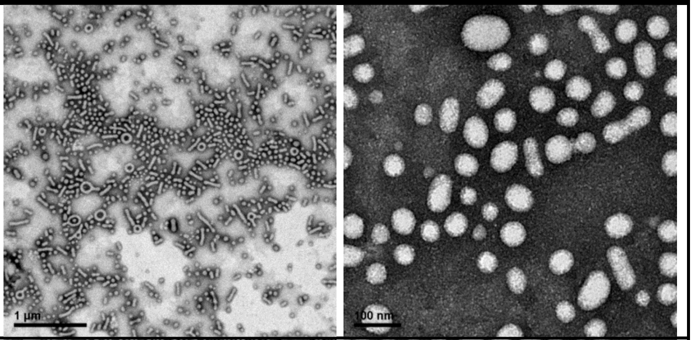
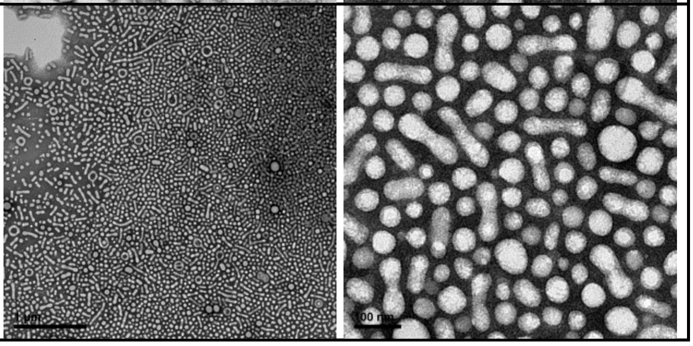
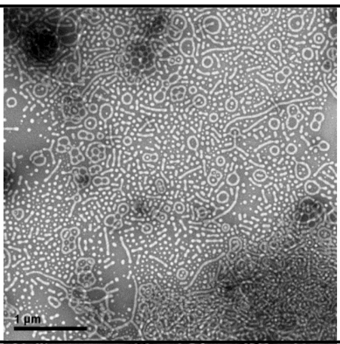
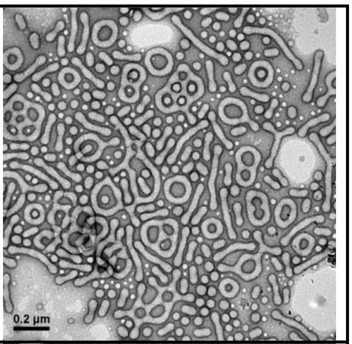
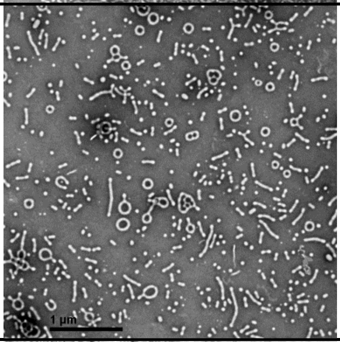
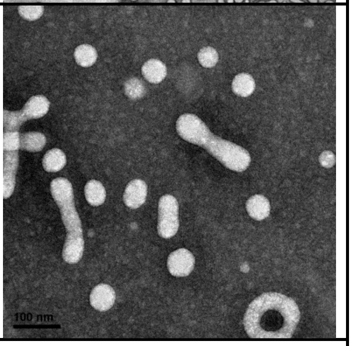
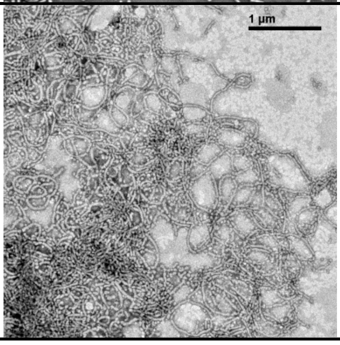


Figure 3.13 TEM images of PEG-b-PDAP aggregates prepared (a) by adding water to a DCM solution and (b) from a DCM/water mixture (see text for experimental conditions).

Encouraged by this last result, new experiments were performed to directly disperse PEG-*b*-PDAP in water, i.e. avoiding the use of an organic solvent. Different experimental conditions of sonication were checked by fixing the time of acoustic irradiation 15 min (3 intervals of 5 min to avoid an excessive heating of the sample) and different working conditions. Clear dispersions were obtained by this methodology, which were studied by TEM to analyze the morphology of the polymeric aggregates. Table 3.2 shows the sonication conditions and the collected TEM images. As can be seen, mixtures of toroidal, worm-like (cylindrical) or spherical micelles are obtained in different proportions under the studied conditions. Although no homogeneous nanoparticles are obtained by this method (in comparison with co-solvent method) the obtained results are really promising taking into account the easiness of this methodology.

Table 3.2. PEG-b-PDAP aggregation in water, with different working conditions.

Conditions (PEG-b-PDAP in Water)	**TEM Image**
70 Amp/ 0.5 Cycle	
90 Amp/ 0.3 Cycle	

90 Amp/ 0.3 Cycle		
90 Amp/ 0.5 Cycle		
90 Amp/ 1.0 Cycle		

Finally, an attempt to apply this methodology to supramolecular BCs was also performed. Unfortunately, a precipitate was finally observed.

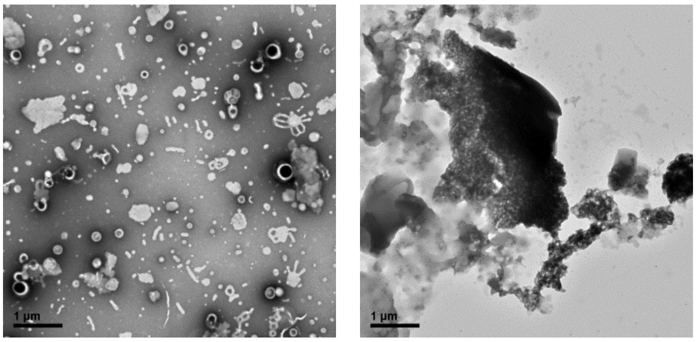


Figure 3.14. PEG-b-PDAP·tAZO 75% aggregation in (a) DCM/water (70 Amp/ 0,5 Cycle/ 5 min + 5 min), and (b) water (90 Amp/ 0,5 Cycle/ 5 min + 5 min + 5 min).

4. EXPERIMENTAL METHODS

4.1 Techniques and Instruments

Infrared Spectroscopy (FTIR). IR spectroscopy was performed with a Bruker Vertex 70. The samples were prepared in KBr pellets (2% wt).

Nuclear Magnetic Resonance (^{1}H NMR). ^{1}H NMR was carried out using a Bruker AV-400 (400 MHz) equipment using $CDCl_3$ as solvent.

Gel Permeation Chromatography (GPC). GPC was carried out using a Waters Alliance 2695 HPLC with an evaporative light scattering detector Waters 2420 and Styragel® Waters columns (7.8 mm ID x 300 mm) HR2 and HR4, using THF as eluent (flow 1 mL/min). Calibration was made with poly(methyl methacrylate) standards. Samples were prepared by dissolving 2 mg of product in 2 mL of THF (HPLC grade).

Thermogravimetric Analysis (TGA). Analysis was registered in a Q5000 from TA Instruments using powdered samples (2-5 mg) from 30 to 600°C at 10 °C/min under nitrogen. The onset temperature was calculated in the first derivative of the TGA curve (DTGA) in the cutoff of the tangent to the mass loss curve and the baseline (related with the beginning of decomposition under the experimental).

Differential Scanning Calorimetry (DSC). DSC experiments were carried on a Q2000 calorimeter from TA Instruments using powdered samples (2-5mg) sealed in aluminum capsules. The scan rate was 10ºC/min. The glass transition temperature, Tg was calculated as the midpoint of the heat capacity jump, and cold crystallization and melting temperature, Tc and Tm were calculated at the maximum or minimum of peak transition.

Ultraviolet-visible spectroscopy (UV-vis). For turbidimetric study, an ATI Unicam, UV4-200 spectrophotometer was employed.

Sonication. A Heilscher Ultrasonic Processor UP400S was employed with a sonotrode for low volumes of aqueous or organic solutions.

Transmission Electron Microscopy (TEM). TEM studies were performed at the 'Servicio de microscopia electrónica' from the 'Servicios Generales de Apoyo a la

Investigación-SAIs de la Universidad de Zaragoza'. A transmission electron microscope high resolution JEOL-2000 FXII was used operating at 200 kV. Sample solution (10µL) is deposited in the copper grid with carbon film (CF400-Cu), after 30 seconds, excess of water and non-deposited sample is removed by capillarity. The sample adhered on the copper grid surface is dyed using uranyl acetate (10µL), after 30 seconds, excess of dye is removed by capillarity. The prepared samples stay 24 h under vacuum to dry before measurement.

Dynamic Ligth Scatering (DLS). DLS measurements were carried out in a Malvern Instrument Nano ZS using a He–Ne laser with a 633 nm wavelength and a detector angle of 173° at 25ºC.

4.2 Synthesis and characterization of the copolymer PEG-b-PDAP

Synthetic Procedure. In a Schlenk flask were introduced 0.283g of DAP (0.75mmol), 0.15g of Macro-CTA, 12.5mL of DMF and 3.10 mg of AIBN. The reaction is maintained under stirring at 80ºC for 5 h. Then, the Schlenk is introduced into liquid nitrogen (quenching of the polymerization) and the crude was precipitated in 100 mL (approx.) of cold diethyl ether. The precipitate is isolated by filtering under vacuum and washed with cold diethyl ether. To purify the compound, the polymer is dissolved in DCM and reprecipitated with cold diethyl ether, in order to remove residual monomer or other impurities that might remain.

Characterization Data

IR (KBr), **ν** (cm-1): 3335 (NH), 1735, 1695 (C=O), 1590, 1525, 1450(Ar), 1150, 1100 (C-O);

^{1}H RMN ($CDCl_3$, 400 MHz), **δ** (ppm): 9.28-8.17, 8.06-7.37, 4.64-3.87, 3.76-3.53, 3.06-2,53, 2,49-1.99, 1,41-0.95.

4.3 Preparation of supramolecular block copolymers

Supramolecular block copolymers were prepared in a vial by dissolving the corresponding amount of block copolymer and tAZO in the minimum volume of

HPLC-grade THF. The solution was filtered using a 0.2 micron PTFE filter and maintained at room temperature under constant mechanical stirring for a gradual solvent evaporation (2-3 days). After this time, the dry-process is finalized in a under vacuum at a moderate temperature.

Characterization of PEG-b-PDAP·tAZO:

* **IR** (KBr), **ν** (cm-1): 3335 (NH), 1735, 1695 (C=O), 1590, 1525, 1450(Ar), 1150, 1100 (C-O),

4.4 Preparation of Self-assembled Aggregates

In order to perform the preparation of polymeric nanoparticles by self-assembly of the BCs, two main methodologies were used: co-solvent and sonication. In each method, the conditions were systematically varied, as it has been explained in this report.

4.4.1 Co-solvent method

5 mg of sample were dissolved in 1 mL of organic solvent (HPLC grade or similar) and filtered using a Teflon filter (0.2 micron). The mixture was transferred to a quartz cuvette where Milli-Q water (or the phosphate buffer) was added in 10 μL portions under gently stirring. This process is followed by UV-vis spectroscopy by measuring the variation of absorbance at λ = 650 nm upon addition and addition of co-solvent was maintained until the absorbance value was stable. Once the nanoparticle formation process is complete, the cuvette content was transferred to a dialysis membrane (pore size 1000Da) to dialyze against Milli-Q water to eliminate the organic solvent. Water needs to be constantly changed every 24h during 4 days.

4.4.2 Sonication

1 mg of sample is weighted in a vial and it is added 1mL of solvent. The vial is placed in the Ultrasonic Processor and the working conditions are chosen. The experimental conditions are widely described in the Results section.

IMPORTANT: to avoid the probe damage is necessary to ensure that the solvent covers at least 1cm of the probe tip.

5. CONCLUSIONS

In conclusion the synthesis of amphiphilic block copolymer was successful accomplished using RAFT polymerization as a suitable method for preparing well-defined BCs.

Polymeric blocks of PEG-*b*-PDAP are miscible in the bulk state and the block copolymer only exhibits a glass transition below room temperature when thermal transitions are studied. DAP units can be easily functionalized by means of hydrogen bonding using thymine-containing functional moieties, in our case with azobenzene chromophores. Upon complexation, polymeric blocks of PEG-*b*-PDAP become immiscible and the resulting supramolecular block copolymers exhibit thermal transitions corresponding to the separated polymeric blocks, in particular when the degree of complexation is higher than 50%.

Co-solvent methodology was successfully applied to the preparation of polymeric nanoparticles derived from PEG-*b*-PDAP. The system THF/water gave good results on the preparation of spherical micelles with a diameter of around 30nm and a good homogeneity according to the TEM micrographs. However, attempts to prepare spherical micelles using supramolecular BCs were unsuccessful and a precipitate was detected in all cases. This precipitate seems to correspond to the azo chromophore.

Sonication was also studied as a methodology for preparing polymeric nanoparticles. PEG-*b*-PDAP was easily dispersed using this method, even without using an organic solvent. Although block copolymer was completely dispersed, micelles with different morphologies were finally observed. This method seems to be highly promising, mainly due to the absence of organic solvent and the easiness of the methodology.

APPENDIX

A.1. ^{1}HRMN PEG-*b*-PDAP

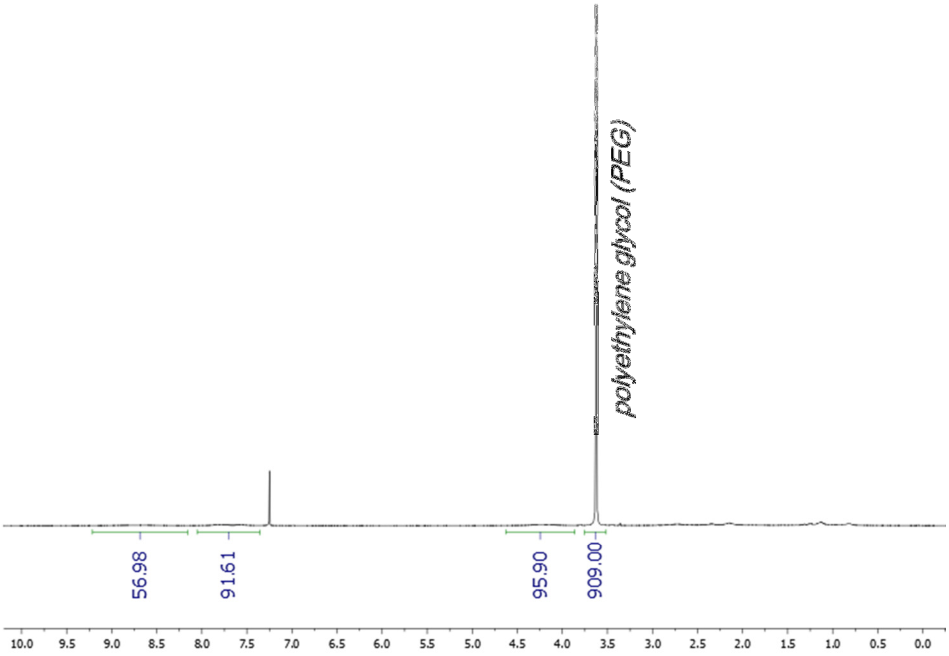


Fig A.1.1. ^{1}HRMN PEG-b-*PDAP (CDCl$_3$, 400MHz) δ (ppm)*

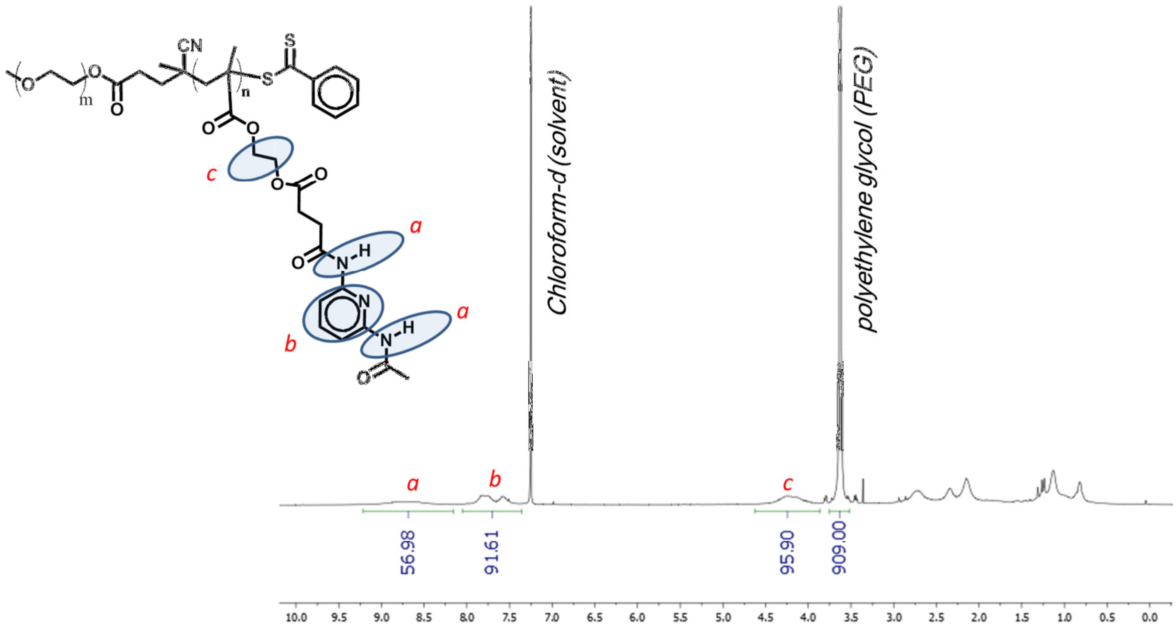


Fig A.1.2. ^{1}HRMN PEG-b-*PDAP (CDCl$_3$, 400MHz) δ (ppm) - ZOOM*

A.2. TGA PEG-*b*-PDAP

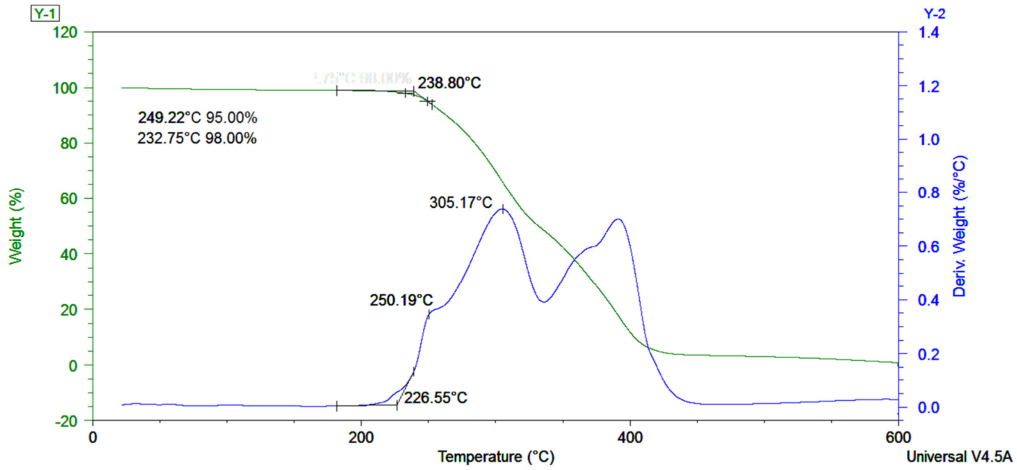


Fig 2.1. TGA Analysis of PEG-b-PDAP.

A.3. FTIR PEG-*b*-PDAP, PEG-*b*-PDAP.tAZO 75%, tAZO

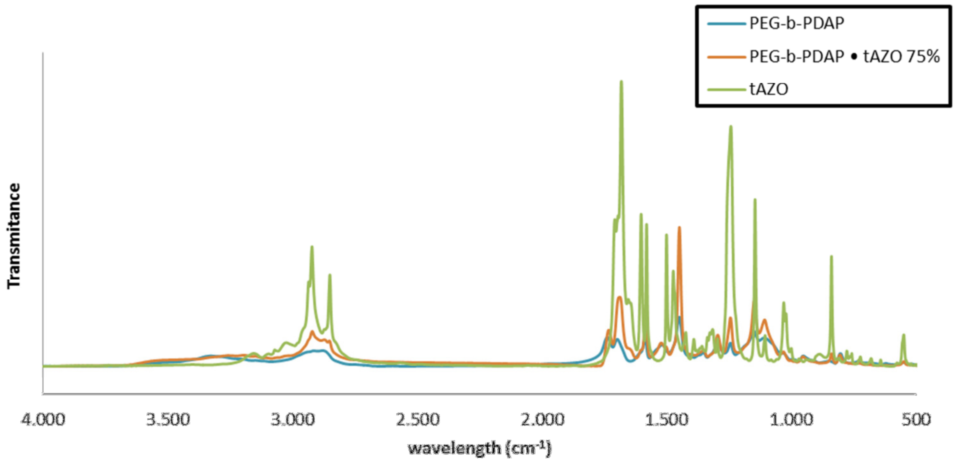


Fig 3.1. FTIR Spectra of PEG-b-PDAP, PEG-b-PDAP·tAZO 75% and tAZO.

A.4. TGA PEG-*b*-PDAP.tAZO x%

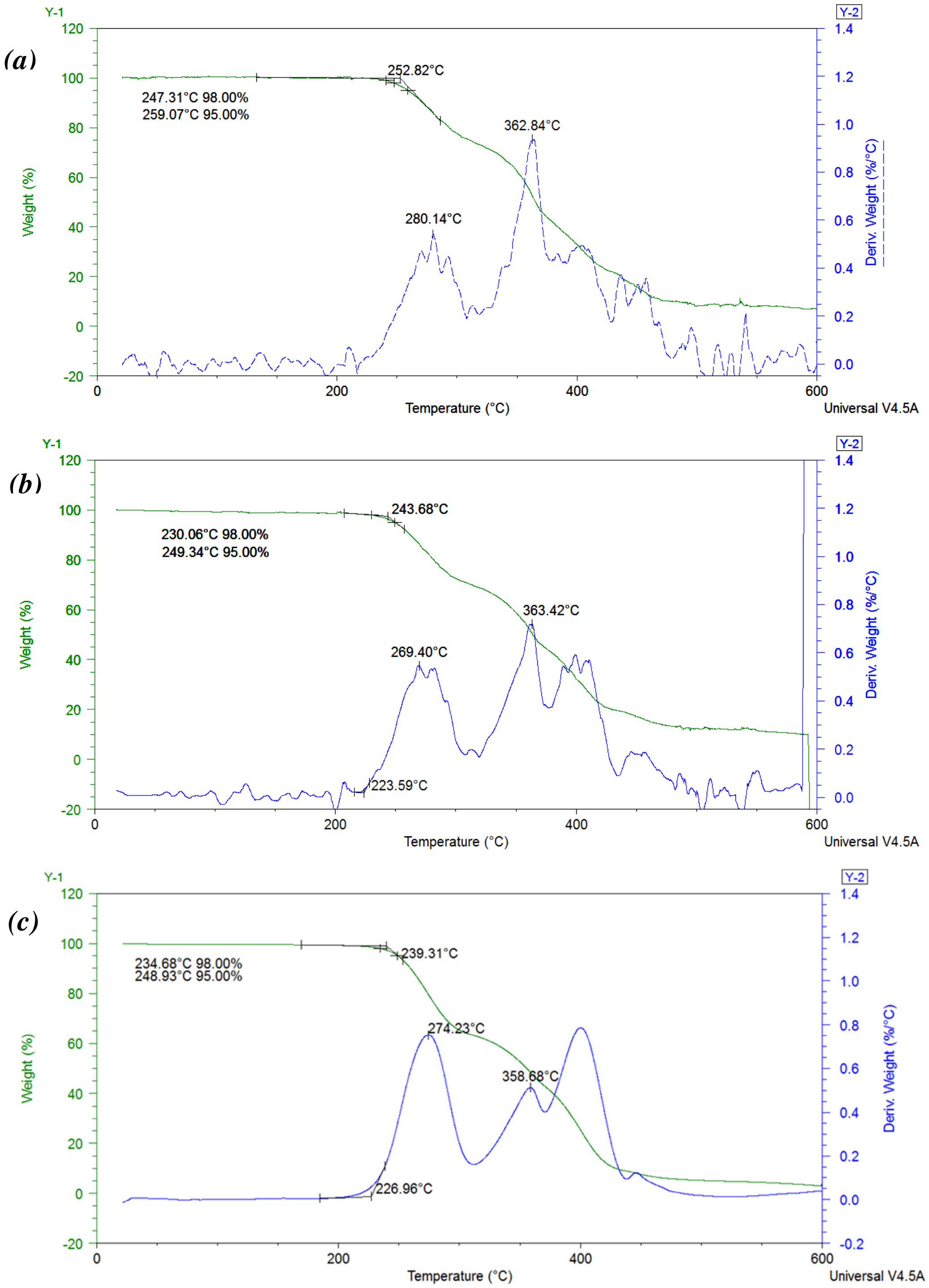


Fig A.4.1. TGA- PEG-b-PDAP·tAZO (a)75%, (b)50% and (c) 25%.

A.5. SIZE Analysis

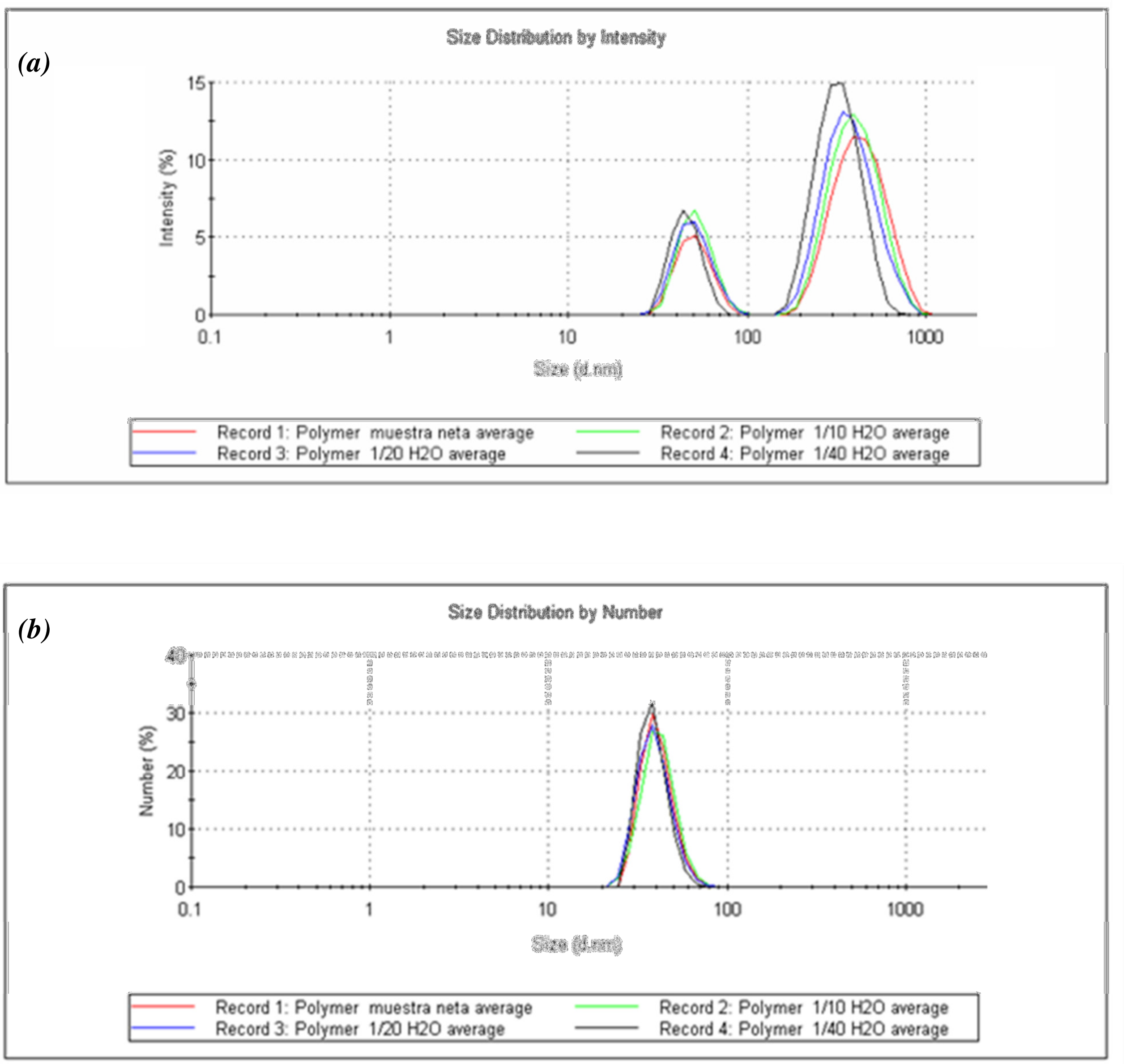


Fig. A.5.1. DLS Analysis of PEG-b-PDAP in function of (a) Intensity and (b) number.

Tabla A.5.1. Average diameter of PEG-b-PDAP from TEM image analysis.

Zone:	I	II	III
Sample	Diameter (nm)		
1	23,12	27,88	24,59
2	26,68	26,31	27,00
3	25,84	27,80	24,75
4	23,34	28,19	27,33
5	27,05	27,13	25,39
6	24,02	26,55	30,43
7	23,14	25,83	28,40
8	24,77	29,28	27,68
9	25,67	26,34	22,81
10	25,09	22,86	27,01
11	22,16	23,72	27,13
12	25,68	25,17	27,01
13	23,34	24,06	22,10
14	28,24	25,67	26,30
15	24,39	26,57	28,43
16	24,58	25,43	21,79
17	23,37	25,35	22,67
18	24,96	25,83	30,57
19	22,10	27,92	31,73
20	24,45	25,62	26,09
Maximum	28,24	29,28	31,73
Minimum	22,10	22,86	21,79
Standard Deviation	1,57	1,55	2,72
Average	24,60	26,18	26,46
Trend	25,05	27,03	26,12

Tabla A.5.2. Average diameter of PEG-b-PDAP·tAZO 75% from TEM image analysis.

Zone:	**I**	**II**	**III**
Sample	Diameter (nm)		
1	43,01	43,84	43,87
2	33,84	46,73	36,53
3	41,44	45,02	36,03
4	37,05	39,60	32,77
5	34,06	44,58	41,11
6	33,42	42,68	41,09
7	37,01	39,42	34,23
8	42,00	42,90	59,89
9	39,81	53,31	38,91
10	36,01	34,21	34,74
11	36,13	34,20	49,39
12	39,25	35,40	36,78
13	35,23	46,27	46,76
14	36,01	33,75	43,33
15	38,01	34,10	38,03
16	37,01	42,52	37,94
17	41,76	44,87	37,56
18	38,86	40,18	38,72
19	39,93	36,85	44,81
20	34,93	51,20	45,65
Maximum	43,01	53,31	59,89
Minimum	33,42	33,75	32,77
Standard Deviation	2,83	5,23	6,24
Average	37,74	41,58	40,91
Trend	37,71	43,84	41,11

Tabla A.5.3. Average diameter of PEG-b-PDAP·tAZO 75% (IR-Irradiated) from TEM image analysis.

Zone:	**I**	**II**	**III**
Sample	Diameter (nm)		
1	44,56	50,14	44,53
2	49,20	43,58	50,36
3	45,87	39,60	56,64
4	40,68	44,13	36,67
5	29,11	40,43	56,67
6	46,45	36,82	43,58
7	50,52	35,56	44,18
8	40,22	33,32	52,67
9	43,53	32,69	40,67
10	47,06	36,72	40,09
11	48,55	40,01	39,36
12	36,16	47,50	49,82
13	34,41	45,65	49,41
14	42,47	39,38	48,37
15	41,80	42,53	40,02
16	37,21	38,05	53,13
17	38,82	29,34	46,21
18	43,90	35,88	47,11
19	37,07	34,90	33,87
20	45,14	37,81	43,68
Maximum	50,52	50,14	56,67
Minimum	29,11	29,34	33,87
Standard Deviation	5,43	5,22	6,34
Average	42,14	39,20	45,85
Trend	44,33	38,95	47,27