

Poly(benzyle glutamate)-based nanoparticles for intercepting and destroying circulating tumor cells into the bloodstream

an Young Taylor Castillo

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Nanoparticules à base de poly(Lglutamate de γ -benzyle) pour l'interception et la destruction des cellules tumorales circulantes dans la circulation sanguine

> Thèse de doctorat en cotutelle de l'Université Paris-Saclay et l'Universidad de Sonora préparée à l'Université Paris-Sud Institut Galien Paris-Sud UMR CNRS 8612

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"Everyone should consider his body as a priceless gift from one whom he loves above all, a marvelous work of art, of indescribable beauty, and mystery beyond human conception, and so delicate that a word, a breath, a look, nay, a thought may injure it."

Nikola Tesla

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(1856-1943)

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In memoriam: Ignacio Taylor

Julieta Castillo

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INTRODUCTION GÉNÉRALE

Introduction

Malgré les progrès continus de la médecine, le cancer reste l'une des principales causes de morbidité et de mortalité dans le monde avec environ 14 millions de nouveaux cas et 8,2 millions de décès liés au cancer en 2014, selon l'Organisation mondiale de la santé. Ainsi, 90% des décès liés au cancer dans le monde sont causés par la propagation de cellules cancéreuses depuis des tumeurs primaires et vers des organes distants dans lesquelles elles s'implantent et sont à l'origine de tumeurs métastatiques. En plein progrès, la détection des cellules tumorales circulantes chez les patients est liée à un mauvais pronostic et à de faibles taux de survie. La propagation des cellules cancéreuses à partir de la tumeur primaire débute certainement dans les premiers stades de la croissance tumorale. L'implantation de tumeurs secondaires à des sites éloignés de la tumeur solide primaire détermine alors souvent la dernière étape de la progression du cancer. La diffusion des CTCs (pour l'anglais « circulating tumor cells ») dans le compartiment vasculaire est un phénomène complexe. Pendant leur circulation la margination des cellules tumorales vers l'endothelium vasculaire est une étape cruciale avant que ces cellules puissent se distribuer dans d'autres organes. On estime que seulement 0,01% des CTCs formeront des tumeurs secondaires. Cependant, leur dissémination est un phénomène continu et il serait intéressant de savoir s'il est possible de bloquer ce mécanisme dès la détection d'une tumeur primaire. Dans ce but, nous nous sommes fixés comme objectif d'utiliser des nanoparticules polymères pour intercepter les CTCs dans le torrent circulatoire. En effet, d'un point de vue biophysique, les CTCs paraissent plus facilement accessibles à des agents thérapeutiques lorsqu'elles sont dans le sang, en comparaison d'autres organes plus compacts que le sang. Le comportement des CTCs dans le sang est de mieux en mieux décrit. Une fois arrivée dans le sang par un mécanisme d'extravasation, les CTCs circulent seules

ou en agrégats pluricellulaires. Les caractéristiques de cette circulation sont alors fortement influencées par plusieurs facteurs tels que la taille, la forme, et la surface. Pour pouvoir intercepter les CTCs dans le sang, les nanoparticules doivent donc posséder des caractéristiques adaptées. Une des caractéristiques importantes est de s'assurer que les nanoparticules seront capables de se concentrer à proximité de l'endothélium vasculaire, où se produisent les phénomènes d'intravasation. Pour assurer cela, il est indispensable de maitriser finement l'architecture des nanoparticules, de manière à assurer simultanément, (i) leur margination au niveau de l'endothélium pour concentrer les particules à ce niveau et ainsi cibler efficacement les CTCs dans les phases d'intravasation ou leur extravasation, (ii) le couplage d'un ou de plusieurs ligands de reconnaissance des CTCs tels que des anticorps ou des polysaccharides, et (iii) d'associer des molécules cytotoxiques à ce système.

Au cours des dernières années, les grandes avancées dans les sciences des matériaux, permettent de concevoir les nanoparticules comme des entités actives par ellesmêmes et non plus seulement comme des transporteurs d'une molécule active. Leur conception et les méthodes qui permettent de les préparer doivent donc être adaptées à cet objectif. Ainsi, la taille, la forme géométrique et la topologie de leur surface sont des caractéristiques essentielles qui déterminent l'architecture des nanoparticules et gouvernent par conséquent leur comportement pharmacocinétique ainsi que leurs interactions cellulaires. Dans l'objectif particulier qui a été le nôtre, c'est à dire le ciblage des CTCs dans le sang, nous avons choisi de développer des nanoparticules constituées de copolymères di- et triblocs du poly(L-glutamate de γ -benzyle) (PBLG) et de poly(éthylène glycol) (PEG). Les blocs PBLG ont été synthétisés par polymérisation par ouverture de cycle de l'anhydride BLG-NCA par la voie des amines. Ces copolymères ont permis d'obtenir des nanoparticules amphiphiles auto-

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morphologies allant de la forme sphérique (rapport d'aspect 1,3) à une forme oblongue (rapport d'aspect 2,6). Ces particules possèdent une organisation anisotrope, avec des zones hydrophiles et des zones hydrophobes. De plus, à l'échelle moléculaire, les chaînes de PEG à la surface sont soit libres (hairy type), soit en boucle (mushroom type). Ces différentes possibilités architecturales ont donc été mises à profit pour préparer des nanoparticules capables de cibler les CTCs dans le compartiment sanguin.

Le présent manuscrit est composé d'une introduction générale, des quatre chapitres et d'une conclusion générale. Un premier chapitre bibliographique a tout d'abord été consacré à analyser l'impact clinique des métastases, l'étape cruciale de la margination cellulaire dans les métastases et les approches actuelles de la nanomédecine pour développer des nanoparticules conçues rationnellement pour faire face au défi des cellules tumorales cibles dans leur voie multidirectionnelle.

Le deuxième chapitre de ce travail décrit la préparation et la caractérisation expérimentales d'une variété de copolymères amphiphiles du PBLG formés par des copolymères di- et tri-blocs. Les blocs PBLG ont été synthétisés avec succès par polymérisation par ouverture de cycle de l'anhydride BLG-NCA par la voie des amines. La conformation α -hélice de la chaîne PBLG de nature hydrophobe, attachée à une chaîne PEG hydrophile courte, a permis d'obtenir des nanoparticules amphiphiles auto-assemblées dont les chaînes de PEG à la surface sont soit libres (« hairy type»), soit de type boucle (mushroom type). Ces nanoparticules ont été préparées par la méthode de nanoprécipitation. Ce chapitre est aussi consacré à l'analyse des propriétés intrinsèques de leur architecture. Ainsi, outre les différences de conformation des chaînes de PEG, ces nanoparticules présentent une importante anisotropie de surface, probablement induite par la rigidité du bloc PBLG.

Le troisième chapitre est consacré à l'évaluation biologique des nanoparticules obtenues. La cytotoxicité, l'activité hémolytique et l'activation du système du complément des nanoparticules amphiphiles auto-assemblées à base de dérivés du PBLG composés par des diet tri-blocs ont été mesurées. L'interaction des nanoparticules présentant diverses morphologies avec des cellules représentatives du compartiment sanguin a également été évaluée quantitativement, ce qui a permis de mettre en évidence que la non-sphéricité (l'élongation) des particules et l'anisotropie de leur surface permettaient de les rendre sélectives de certains types cellulaires.

Le quatrième chapitre décrit la préparation d'immunonanoparticules conjuguées avec l'anticorps MART-1. Cet anticorps reconnait l'antigène spécifique surexprimé dans la membrane et le cytoplasme des cellules métastatique de mélanome, pour lequel il existe des modèles intéressants mimant le développement métastatique.

Par ailleurs, l'évaluation de leur comportement *in vitro* par l'activation du complément, la mesure de la cytotoxicité et la mesure de l'intensité des d'interactions cellulaires sont présentées. La conjugaison de cet anticorps sur la surface des nanoparticules leur confère des propriétés de ciblage pour l'antigène surexprimé dans les cellules de mélanome. Les résultats suggèrent que les nanoparticules à base de PBLG couplées à l'anticorps MART-1 sont des outils intéressants pour assurer le ciblage des cellules du mélanome. Les voies d'optimisation des paramètres importants de ces immunonanoparticules sont discutées.

Enfin, une discussion générale reprend l'ensemble des résultats expérimentaux afin de les présenter de manière synthétique et aussi de façon à les mettre en perspective avec des travaux décrit dans la littérature.

TRAVAUX ANTÉRIEURS

CHAPITRE I

CIRCULATING TUMOR CELL, NANOTECHNOLOGY SCOPE FOR

METASTASIS TREATMENT

CIRCULATING TUMOR CELL, NANOTECHNOLOGY SCOPE FOR METASTASIS TREATMENT

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Abstract

Despite outstanding improvements in medicine, cancer is one of the leading causes of morbidity and mortality worldwide with approximately 14 million new cases and 8.2 million cancer related deaths in 2014, reported by the World Health Organization. 90% of cancer-related deaths worldwide is produced by the spread of cancer cells toward distant organs. In fact, the detection of circulating tumor cells in patients is related to poor prognostic and low survival rates. In this review, it will be analyzed the clinical impact of metastasis, the crucial step of cell/particle margination in metastasis process and the currently approaches of nanomedicine to develop rational designed nanoparticles to face the challenge of target circulation tumor cells in their multidirectional pathway.

Keywords: metastasis, circulation tumor cells, nanomedicine.

Introduction

Cancer is a disease in which its scope goes beyond the patient. It is impacting patient's family, friends and overall the society. For decades, the fight against cancer has reached several fields in science, as a global effort not merely to treat it but also to understand how deeply rooted is the problematic even beyond patient's remission. It is encouraging to develop different approaches to ensure social wellness and to improve the current strategies in cancer therapy in a broad sense.

In fact, it is well-documented that patients and close entourage, face important repercussions of cancer that are not merely physical (e.g. fatigue, pain, morbidity, fertility limitation) (Portenoy *et al.*, 1999; Curt *et al.*, 2000; De Vos *et al.*, 2014; Chiles *et al.*, 2016), but financial (Ramsey *et al.*, 2016; Buzaglo *et al.*, 2017) and mentally (e.g. depression, anxiety, adaptation, etc.) (Ell *et al.*, 1988; Edwards *et al.*, 2004). Indeed, even with the remarkable improvement in medicine, cancer is one of the leading causes of morbidity and mortality worldwide with approximately 14 million new cases and 8.2 million cancer related deaths in 2014, reported by the World Health Organization. The most concerning aspect is that nearly 90% of those cancer deaths are related directly to tumor spread in distant organs, referred to as metastasis (Gupta and Massagué, 2006).

The complex progression of metastasis is englobed in five broad steps. Once established the solid tumor, cancer cells from either primary tumor or metastases, develop the capacity to up-down regulate genes to dissociate from the solid tumor, followed by the invasion through the extracellular matrix. After a successful invasion of the surrounding tissue microenvironment, tumor cells intravasate through the bloodstream or lymphatic flow to disseminate in distant sites. The tumor cells in the intravasation process are denominated circulating tumor cells (CTCs). Once in a favorable environment, the CTCs actively leave the vasculature to colonize and form secondary tumors. In addition, some successful CTCs once in the colonized site could remain "silent" for many years, which behavior is denominated dormancy (Gupta *et al*; 2006; Chaffer *et al*., 2011; Alizadeh *et al*., 2014).

The poor survival outcome of patients with metastasis is related to existence of the heterogeneous cell subpopulations produced after their adaptation process. Despite that a substantial amount of CTC may be unsuccessful in establish secondary tumors, the remaining CTCs will present different metastasis potential that is generally highly aggressive (Jacob *et al.*, 2007; Bertazza *et al.*, 2008). In fact, it is considered that only 0.01% of CTCs will successfully form secondary tumors (Hong and Zhang, 2016). Thus, in this work, we review the clinical importance of CTCs in metastatic process and discuss the current nanomedicine approaches for CTCs treatment.

Circulating tumor cells, metastasis tumor progression

In the scope to understand the complex process of metastasis, several theories have been proposed to explain the selectivity of tumor spread and the tumor progression into metastasis. The seed and soil theory, been proposed by Stephen Paget in 1889, is regaining interest due to experimental observations. This theory suggests that despite circulating tumor cell are released in all directions, only those that find the correct microenvironment or "soil" will establish secondary tumors (Chambers *et al.*, 2002; Fokas *et al.*, 2007; Jacob *et al.*, 2007). As an example of this theory, it is observed in breast cancer metastasis, a remarkable preference for bone and lungs over other organs like brain and liver. This is correlated with observations of the constant interaction of CTCs into those sites. The stromal components in the bone and the primary tumor induction of matrix metalloproteinase 9 (MMP9) expression in lung endothelial cells combined with the prior recruitment of tissue-associated macrophages could promote the successful 'soil' for metastasis progression (Weigelt *et al.*, 2005).

Furthermore, the induction or adaptation of pre-existing physiological sites in distant organs, hypothesized as pre-metastatic niches, is remarkably complex. In the initiation of premetastatic niche, haematopoietic progenitor cells are implicated to secrete factors, chemokines, and matrix-degrading enzymes, that will recruit other inflammatory cells, endothelial progenitor cells and mesenchymal cells to mediate the chemoattraction to the niche, enhancing CTCs adhesion and survival (Psaila and Lyden, 2009). Indeed, high levels of transforming growth factor beta (TGF- β) cytokine and chemokine (C-X-C motif) ligand 1 (CXCL1) have been associated to the presence of clustered CTCs in aggressive metastatic colorectal cancer (Divella *et al.*, 2014).

Epithelial-mesenchymal transition (EMT) plays a key in tumor progression. EMT has been associated with the formation of CTCs and the induction of invasive capacity. EMT is defined as a reversible process that switches epithelial phenotype to a fibroblastoid or mesenchymal cellular phenotype. (Liu *et al.*, 2015; Hong and Zhang, 2016). The reversible transition of EMT is called the mesenchymal-epithelial transition (MET) and has been hypothesized after the CTCs extravasation step (Hong and Zhang, 2016). The EMT/MET process is mediated by the abnormal activation of genes that down-regulate the epithelial markers such as E-cadherin and cytokeratins, as well as up-regulate the mesenchymal markers such as vimentin, N-cadherin, and matrix metalloproteinases (MMP) (Savagner, 2001; Liu *et*



al., 2015). These markers are involved in the cell-cell interactions and the arrangement of the cytoskeleton (Savagner, 2001).

The suppression of E-cadherin is related to tumor progression and poor survival. Ecadherin is a type-1 cadherin within a large family of transmembrane glycoproteins that mediate specific cell-cell adhesion in the epithelial cell behavior (Van Roy and Berx, 2008). It has been observed that the activation of the Snail family transcription factors has a great impact on the suppression of E-cadherin and the triggering of EMT transition (Mikami et al., 2011). Within the large Snail family, Snail (Snail1) and Slug (Snail2) transcription factors are closely related with the loss of cell adhesion and the acquisition of migratory and invasive properties by cells (Kurrey et al., 2009). Several Snail and Slug expressions evidence are found in the literature as major determinants of tumor progression by MET transitioning. Mikami and colleagues analyzed the association of Snail and Slug expression with cancer invasion and prognostic in renal cell carcinoma (RCC) (Mikami et al., 2011). Their research was based on the expression and migration capacity of RCC cell line 786-O, after treatment with a small interfering RNA (siRNA) to down-regulate Snail gene expression. The treatment with the siRNA not merely down-regulate the gene expression of Snail but also the vimentin, MMP2 and MMP9 expression in 786-O cells (that are also related with tumor progression) and upregulate the expression of E-cadherin. Furthermore, it was also positively found that the expression of Snail, MMP2, and MMP9 are correlated with pathological tumor stage and the presence of sarcomatoid carcinoma. In contrast, it was found that Slug protein and their genes expression were down-regulated in advances RCCs. Based on the results obtained by Mikami and colleagues, the expression of Snail, MMP2 and MMP9 could be indicators of tumor progression and worse predictors of disease survival of RCC patients.

The expression of Snail and Slug was also analyzed by Kurrey and colleagues in the cell line SKOV3 corresponding to ovarian cancer (Kurrey *et al.*, 2009). They observed that Snail and Slug expression enhanced the EMT transition, motility and invasiveness by loss of intracellular adhesion, alterations of desmosomal and tight junction components. Those results are correlated with high aggressiveness of epithelial ovarian carcinoma. Furthermore, it was also observed that Slug is expressed in adverse conditions. Kurrey and colleagues concluded that in the initial steps of cell detachment from the primary tumor due to the hypoxia conditions, the expression of Slug down-regulate the E-cadherin and other adherent components for a brief period followed by the expression of Snail, that is the major transcriptional repressor of E-cadherin. Therefore, Snail and Slug could have distinct roles in tumor progression (Kurrey *et al.*, 2009; Liu *et al.*, 2015).

It has been observed that the activation of Snail and Slug expression, could be trigged through different pathways. Zhao and colleagues (Zhao *et al.*, 2007) analyzed the role of LIV-1 in the regulation of Snail expression in cervical carcinoma. LIV-1 is part of the ZIP family of zinc transporters as an integral plasma membrane protein. They found that the transcription level of LIV-1 is particularly higher in cervical cancer *in situ* but not significant difference was detected compared with normal and invasive cancer tissues. However, as zinc is a component of DNA and RNA polymerase, their results suggested that LIV-1 is associated with HeLa cells growth, but the suppression of LIV-1 has no effect on cell apoptosis. Instead, the protein sequence exhibits a potential metalloprotease motif that once knocked out in HeLa cells, the expression of Snail and Slug is reduced, and the cancer migration and invasion potential are inhibited. This remark could suggest that LIV-1 may regulate Snail expression since it is associated with EMT transitions. Furthermore, Taylor and colleagues (Taylor *et al.*, 2003),

also found a relation between LIV-1 and tumor spread. They evaluated the implication of its expression in breast cancer progression. They found that LIV-1 gene expression could be stimulated by oestrogen treatment and therefore, it could be associated with oestrogen receptor status. Their results also exhibited that LIV-1 mRNA expression is importantly associated with breast cancer spread to regional lymph nodes, in where they might grow. However, since breast cancer cells in the lymphatic nodes would enter to blood circulation at a certain point to arrive at those sites (Chambers *et al.*, 2002). As founded by Zhao and colleagues, the metalloprotease motif similarity of LIV-1 compared with the matrix metalloproteases (MMPs) could play a significant role in the metastasis of breast cancer cells. Moreover, it is the association of LIV-1 with ubiquitin-dependent degradation, a small molecule that could affect proteins through degradation, alteration of their cellular location and their activity, that could contribute to developing cancer/metastasis and it could be considered as a marker of cancer progression (Zhao *et al.*, 2007).

Regarding the alternatives pathways of EMT transition activation, it is documented that Twist proteins could also stimulate EMT transition through their role in cell migration promotion (Liu *et al.*, 2015). Twist proteins are part of the basic helix-loop-helix (bHLH) transcription family, their characteristic conformation allows them to bind with the hexanucleotide sequences of DNA 5'-NCANNTGN-3' referred to as E-boxes. Those E-boxes are elements of the regulatory gene DNA information. Therefore, they can act as positive or negative regulators (Puisieux *et al.*, 2006). bHLH transcription factors are organized into three major categories: Class A bHLH factors that include E2-2, HEB and two isoforms of E2A gene E12//E47; the Class B bHLH factors that are involved in the tissue-restricted regulators and the inhibitory HLH protein by Id proteins. Among the bHLH factors twist proteins form a subfamily that includes Paraxis, Scleraxis, Hand1, Hand2, Twist1 and Twist2 (Franco *et al.*, 2011). Specially, Twist proteins promote cell migration through their capacity to down regulate the expression of E-cadherin and the up-regulation of mesenchymal markers. In addition, it has been reported that Twist proteins could protect cancer cell from apoptosis Myc-, p53-dependent, and p53-independent pathways. As proto-oncogenes, Twist1 can up-regulate the protein kinase, AKT2 modulator, that is involved in cell proliferation. Twist proteins combined with E12 could directly also regulate p21, that is involved in growth arrest (Maestro *et al.*, 1999; Puisieux *et al.*, 2006; Franco *et al.*, 2011). As Twist proteins could be attractive for the development of cancer therapy.

As a part of the EMT transition, the rearrangement of cell shape is a crucial phenomenon existing specially in the migration step. It has been observed in migrating cancer cells an overexpression of Vimentin. As a part of the intermediate filament (IF) family proteins, Vimentin is a type III intermediated filament protein and one of the most expressed proteins of IF family in mesenchymal origin cells such as fibroblast, epithelial cells, leukocytes, among others (Satelli and Li, 2011; Dave and Bayless, 2014). The role of IF proteins is associated with adhesion, migration, and regulation of the cytoskeleton integrity. Therefore, the overexpression of vimentin is correlated with increased motility and invasiveness capacities in cancer cells, since is has been demonstrated that their networks are highly dynamic. It is often used as a marker of EMT since their expression is tissue type specific and its detection is predicting poor diagnostic in patients (Dave and Bayless, 2014; Lowery *et al.*, 2015). In fact, Leader and colleagues, analyzed the role of vimentin as a tumor marker (Leader *et al.*, 1987). They

evaluated a variety of cancer cell types, in which it can be found 198 sarcomas, 38 carcinomas, and 22 melanomas. They found an important impact as a useful tool of vimentin commercial antibody to separate sarcomas from carcinomas with some exceptions, distinguish melanomas from carcinomas, and furthermore, combining vimentin commercial antibody with cytokeratin antibodies, they identify synovial sarcomas and carcinosarcomas. Those results highlight the importance of vimentin as a tumor marker and diagnostic tool.

In the early stages of tumor progression, it is demonstrated that solid tumors cannot grow further than 2 mm in diameters without developing angiogenesis (Folkman, 1971). The angiogenesis is referred as the formation of neo-vasculature from preexisting vessels (Banerjee *et al.*, 2011). The high demand for oxygen and nutrients stimulates the apparition of uncontrolled angiogenesis. That phenomenon leads to leaky and intricate blood vessels, that are constantly under inflammatory state, condition that is associated with metastasis, tumor recurrence and poor survival rates (Banerjee *et al.*, 2011; Yadav *et al.*, 2015). Combining the loose union in the epithelial membrane, the direct access to circulation and the tumor progression of cancer cells, it has been estimating by model systems that approximately 1 x 10^6 tumor cells/g of tumor tissue could be shed daily either in the bloodstream or in the lymphatic system (Chambers *et al.*, 2002; Bertazza *et al.*, 2008). It is well-known that the formation of angiogenesis is a key point in establishing new tumors.

The machinery that is used in tumor progression plays a crucial role in embryonic development, wound healing and regeneration, but it is their aberration activation that allows to cancer cells to growth and migrate to distant organs and form secondary tumors. Despite that several pathways in tumor progression remain unclear, the remarkable advance in elucidating
the transition from a normal cell into a cancer cell it is providing new hallmarks for cancer therapy innovation.

Clinical impact of Circulating Tumor Cells

According to experimental results, circulating tumor cells are rarely detected in healthy patients. In fact, Allard and colleagues (Allard et al., 2004) performed an extensive analysis of healthy, non-malignant and carcinoma metastatic patients. The analysis was executed through CellSearch system to detect circulating tumor cells in 7.5mL of blood sample. They observed an extended negative detection in healthy/non-malignant patients in contrast to those undergoing cancer. The number of CTCs detected ranged from 0 to 23,618 CTCs per sample with broad heterogeneity within carcinoma. It is inferred that the presence and number variation of CTCs observed could be associated to the level of tumor vascularization, metastasis placement and aggressiveness degree (Yilmaz et al., 2007; Divella et al., 2014). Thus, since CTCs shed from the primary tumor could be observed in early steps of tumor growth, the development of detection techniques for CTCs could be considered as a real-time measurement of tumor behavior (Alix-Panabières and Pantel, 2013; Diaz and Bardelli, 2014). In fact, Cui and Colleagues (Cui et al., 2015) determined the prognostic value of CTCs and disseminated tumor cells (DTC's) in ovarian cancer patients. Their meta-analysis includes 16 studies, meaning 1623 subjects and the calculation of different parameters such as Odds ratio (OR), overall survival (OS), progression free survival/disease free survival (PFS/DFS) and Hazard ratio (HT). They found through their analysis that the detection of CTC/DTC has a potential predictive value for OS and PFS/DFS parameters. The cases where the CTC/DTC were related with the clinicopathological characteristics of advanced tumor stage, could be associated with poor prognostic and high incidence of tumor recurrence.

As the metastasis process is taking place in tumor progression, the up-down regulation of oncogens confers to circulating tumor cells different capacities that impact the patient prognostic and hallmark the importance to develop a personalized therapy. The inner correlation of Snail and slug activation with EMT transition, allows to the cancer cell not merely to acquire migration capacities, but also to acquire resistance to cell death, radioresistance, and chemoresistance, to enlist some. Vega and colleagues (Vega *et al.*, 2004), found that Snail-expressing cells are resistant to TGF- β -induced cell death and protected from pro-apoptotic factors due to the activation of Mel/Erk and P13K/Akt survival pathways and EMT transition.

As well as Snail, Twist 1 and Twist 2 proteins could overpass oncogene-induced senescence as determined by Ansieau and colleagues (Puisieux *et al.*, 2006). They used an MMTV-ErbB2/NEU transgenic mouse model to develop focal mammary tumors that metastasize to the lungs. They selected Twist 2 positive and negative cells from the tumors to treat them with RNA interference. They observed that the inhibition of Twist2 in Twist2-expressing cell is followed by the trigger of cellular senescence. Moreover, it was also observed that Twist 1 and Twist 2 proteins can override oncogene-induced premature senescence through the elimination of crucial regulators of P53- and Rb- pathways.

Metastasis is generally accepted as a sort of unidirectional spread of cancer cells to distant sites. However, different hypothesis and experimental evidence demonstrate that metastasis is not merely cells seeding but also a self-seeding phenomenon. Norton and Massagué began to address the issue of self-seeding (Norton and Massague, 2006). They hypothesized that tumor growth and metastasis could be considered as a continuous selfseeding that could take place whether at distant sites or within the near tumor environment. Considering that tumor growth is limited by its volume and as EMT is taking place, the primary tumor could release CTCs in the tumor microenvironment that could seed itself, creating a conglomerate of solid tumor masses. Additionally, the CTCs that successfully intravasate to bloodstream could seed distant organs. This behavior could exist from the primary tumor to itself (Self-seeding), to distant metastasis (primary seeding) or from metastasis to the primary tumor (secondary seeding).

Recently, Comen and colleagues (Comen and Norton, 2012) have described a selfseeding model in breast cancer. They suggested that CTCs could enter easily into the primary tumor due to less rigorous barriers (leaky vasculature and tissue-specific factors) and then, follow an S-shaped Gompertzian growth curve. Suggesting that a primary tumor could turn from a unique mass to a conglomerate of continuous masses. Those masses will grow as a function of surface area as opposed to volume, since the CTCs are primarily in the surface and consider that self-seeding CTCs are often highly aggressive phenotype expressing MMP1/collagenase-1, actin cytoskeleton component fascin-1 and CXCL1 that could boost tumor growth, angiogenesis and recruitment of myeloid cells. (Comen *et al.*, 2011). Following this approach, Scott and colleagues have proposed a mathematical model for tumor self-seeding in which they suggest secondary metastatic deposits as a part of primary tumor growth (Scott *et al.*, 2013).

An extended experimental evidence of self-seeding phenomenon was demonstrated by Kim and colleagues (Kim *et al.*, 2009). They used several cancer cell lines such as breast carcinoma, colon carcinoma, and malignant melanoma. Their work was performed under different conditions to evaluate parameters such as cell seed, tumor seed, infiltration capacities, cell attraction and tumor-derived mediators, promotion of tumor growth and self-seeding. The results provided valuable information of metastasis process. They found that tumor models became rapidly seeded in a multidirectional pathway, either as self-seeding or cross-seeding from other cancer subtypes cell inoculated (cases of mammary and melanoma tumors). This evidence could explain the relapse of tumor reseeding despite tumor removal. The re-infiltration processes of CTCs are guided by different biological functions and could depend on tumor type. Also, the authors remark that self-seeding requires almost non-additional adaptation. However, only the CTCs population that have a full complete metastatic function will succeed in seeding, that normally is highly aggressive CTCs type. The selection that undergoes in seeding with highly aggressive CTCs is related to their capacity to interact with the tumor stroma resulting in a release of signals that enhance tumor growth, angiogenesis, invasion, and metastasis.

Considering that CTCs can be released in early steps of tumor growth and could seed either the primary tumor or distant metastasis, their detection in cancer patients is related to poor prognostic and survival. Therefore, the characterization of highly aggressive phenotype CTCs and their migration process could guideline and encourage therapy innovation not merely to CTCs in the presence of tumor mass but also as residual neoplastic cells.

Nanotechnology for circulating tumor cell. Rationally designed nanoparticles, the key approach?

Despite many advantages, current therapies consisting in the direct administration of small drug molecules into the body may also have severe drawbacks. In many cases, the lack of organ specificity and other pharmacokinetics limitations deflect substantial drug amounts to unwanted sites where they can exert important side effects. Thus, to reach the target site with

the effective therapeutic concentration, the dose has to be maintained by repetitive administration of high drug doses. While providing an adequate therapeutic efficacy, this situation could exacerbate side effects, depending on the drug.

To overpass those obstacles, nanoparticles have emerged not merely to prevent drug degradation or enhance drug solubility, but also to address a precise target (Couvreur et Vauthier, 2006; Kingsley *et al.*, 2006; Coelho *et al.*, 2010; Kou *et al.*, 2013; Blanco *et al.*, 2015). Various types of nanoparticles have been imagined attempting to improve the PK profile of various drugs, mostly small molecules. Metal-polymer nanoparticles (Wen *et al.*, 2013), triblock polymeric nanoparticles (Zhang and Zhuo, 2005) and micelle-polymeric nanoparticles (Ko *et al.*, 2009; Zhang *et al.*, 2011; Navarro *et al.*, 2015) are some examples of the revolution in drug delivery systems, since the introduction of the "magic bullet" concept, theorized by Paul Ehrlich in 1891 (Cooper, 1964).

In recent years, considering the great advance in material science, the design of nanoparticles in the general frame of nanomedicine, is transitioning from the aim to include several "basic" properties (e.g. drug encapsulation, protection, release) into a more rational design. This transition is mainly focused on the customization of particles for gaining in specificity. It implies to impart a precise architecture to the nanoparticles, including size, shape and surface characteristics, to modulate their fate in the body (Duan and Li, 2013; Toy *et al.*, 2013; Jun Deng and Changyou, 2016; Toy *et al.*, 2016).

In the literature are found several examples of how a rational design can dramatically affect nanoparticle performance once delivered. In terms of size, the nanoscale range is defined from 1 nm to 1,000 nm. However, it is found that nanoparticles within 20-200 nm could have



an appropriate size for intravenous administration (He *et al.*, 2010; Albanese *et al.*, 2012; Elsabahy and Wooley, 2012; Sykes *et al.*, 2014). Nanoparticles with small size (<10 nm) filtered by lung, kidneys, liver or spleen (Chambers and Mitragotri, 2004; Alexis *et al.*, 2008; Upponi and Torchilin, 2014; Toy *et al.*, 2016), while particles above 200 nm could be easily captured by the reticuloendothelial system (RES). In addition, nanoparticles within the optimal size can benefit from enhanced permeability and retention effect (EPR). First described by Matsumura and Maeda (Matsumura and Maeda, 1986), EPR phenomenon is based on the non-specific accumulation through the leaky vasculature and poor lymphatic drainage in the tumor (Bazak *et al.*, 2014). Among their results, Matsumura and Maeda also described that small molecules under 30 kDa, such as neocarzinostatin (12kDa) do not exhibit EPR effect (Matsumura e Maeda, 1986; Matsumura *et al.*, 1987; Maeda, 2012; Upponi and Torchilin, 2014). Therefore, the optimal size of nanoparticles needs to be carefully looked for to avoid fast elimination.

Nanoparticles shape has recently attracted attention as a neglected parameter so far. Interestingly, it has been suggested that non-spherical particles could undergo a margination phenomenon. This phenomenon occurs in the blood flow when a non-spherical nanoparticle which is subjected to hydrodynamic forces, is deflected towards the vessel walls, due to heterogeneous distribution of hydrodynamic forces. At the opposite, the forces are homogenously distributed on a spherical nanoparticle, which result in a preferential localization of the nanoparticle in the blood flow. Size effects are similarly observed, for which large sizes remain also strapped in the blood flow, rather than exploring the surface of the blood vessels. From a biophysical standpoint, in elongated nanoparticles, the forces, heterogeneously distributed, generate a rotational and translational movement, allowing the nanoparticle to escape from the center of the flow towards the vessel walls (Doshi *et al.*, 2010; Thompson *et al.*, 2013; Toy *et al.*, 2013; Ponchel and Cauchois, 2016).

The role of margination become more evident when the internalization of nanoparticles is necessary to reach the target. In fact, Jurney and colleagues (Jurney et al., 2017) explored the role of the shape of nanoparticles on their cellular uptake in dynamic conditions. The author used non-charged poly(ethylene glycol) (PEG) hydrogel nanoparticles in the shape of rods (~800 and ~400 nm) and disks (~325 and ~220 nm) and endothelial cells, respectively. They compared the static and dynamic cellular uptake conditions of the four rod/disk and spherical nanoparticles. They found that larger nanoparticles were more uptake than those with smaller sizes regardless if those were rods or disks. The authors attributed those results to two forces. In larger nanoparticles, the gravitational force plays a decisive role in the margination and adhesion of them in the bottom surface. In contrast, in the smaller nanoparticles, the Brownian force lead the margination over gravitational force. Also, the authors remark that the gravitational force is reciprocal to the density difference between the particles and the medium. Therefore, those with a higher density could marginate and adhere further to the bottom of the channel. However, the results also exhibit that the margination is highly influenced by other factors as it was observed in the case of 400 nm rods that do not show an increased uptake as compared with the 220 disks of similar volume did. The challenge of designing nanoparticles exhibiting desired architectures for drug delivery was approached by Liu and colleagues through an analysis of the computational modeling tools available (Liu et al., 2012). The authors analyzed how computer simulation could help the optimization of nanoparticles design. Particularly, the authors approached through theoretical models the impact of nanoparticle shape on the adhesion probability. They suggested that non-spherical nanoparticles, meaning

oblate, rod and disc shaped particles showed higher adhesion probabilities than spherical ones due to the dislodging force and the nanoparticle volume, based on the probabilistic kinetic analysis used in McQuarrie and Decuzzi model.

Considering the impact of particles shape on their cellular capture, experimental data are somewhat contradictorily. It is reported in the literature that elongation ratio can hinder the cellular uptake. If the elongated nanoparticle is facing with the long axis the cell membrane, the obstruction is related with the greater time to wrap the nanoparticle. In contrast, if it is facing with the minor axis or present a spherical shape, the cellular uptake is favored (Champion *et al.*, 2007; Verma and Stellacci, 2010). In contrast, nanoparticle shape could facilitate the cellular uptake through active targeting (e.g. antibodies, proteins, etc.). The elongated nanoparticles could present the ligands in a parallel orientation, making relaxed bindings with higher interaction points that otherwise, in the case of spherical nanoparticles due to the circular angle could be tensioned (Thompson *et al.*, 2013; Toy *et al.*, 2013).

In the case of CTCs, the process of migration from the primary tumor is highly complex, and the question, "where will CTCs be found?", remains a challenge for nanoparticle rational design. The malignant cells are released from the primary tumor and travel in the bloodstream, then extravasated and internalized in a new site, till reaching the correct microenvironment, (Chambers *et al.*, 2002). At first sight, elongated nanoparticles could have more exploration capacities since their movement will allow a higher displacement through the vessel. Although, if the malignant cell remains captured mostly in flow, a semi-elongated nanoparticle could have more time to interact with CTCs. Considering the experimental evidence, the general architecture or rational design of novel nanoparticles need to be further analyzed and once mastered, it will be a great advantage in the development of drug delivery systems.

Among the intrinsic characteristics of nanoparticles, the hydrophilic/hydrophobic and nature of the surface as well as the electric charge, influence substantially the nano-bio interface. The membrane cells with an anionic nature seem to spontaneously adsorb nanoparticles with positive ζ potential or hydrophobic nature (Duan and Li, 2013). However, the analysis of this behavior is still complex. Nanoparticles with those characteristics also exhibit spontaneous aggregation and an important level of immunological recognition, situations that limit their effectiveness to reach the tumor site (Duan and Li, 2013). To avoid those situations, the hydrophilization of the surface with a protective layer of poly(ethylene glycol) (PEG), a highly flexible, neutral and hydrophilic polymer that is widely used to avoid opsonization by serum proteins and recognition by the immunological system through steric hindrance (Hu et al., 2007; Li and Huang, 2010). Also, another approach is to include a surfactant into the preparation process. A commonly used surfactant is poloxamer, a triblock copolymer in ABA conformation, that is strongly adsorbed on hydrophobic surfaces, leading to the formation of a protective layer of PEG (Alexandridis and Alan Hatton, 1995; Santander-Ortega et al., 2006). The advantage of this layer it not merely to enhance circulation half-life but also to functionalize the surface with antibodies, protein or molecules of interest (Kou et al., 2013).

Nanomedicines for circulating tumor cell treatment

So far, in the field of CTCs nanotechnology have been mostly dedicated to detection, enrichment, and characterization of CTCs *ex vivo* from blood samples. Although some of those technologies make use of nanoparticles, only a few works are found in the literature as a potential treatment of CTCs. As it will be review in detail, the following works are great examples of currently approaches to treat metastasis.

As a strategy to block tumor dissemination, Mitchell and colleagues formulated TRAIL-coated leukocytes to kill circulating cancer cells (Mitchell *et al.*, 2014). Considering that TRAIL is a tumor necrosis factor (TNF)-related apoptosis-inducing ligand and E-selectin (ES) is an adhesion receptor, they prepared coated ES/ TRAIL liposomes, which were used as tools making possible the functionalization of leukocytes with ES/ TRAIL ligands. This transfer was achieved under controlled shear flow conditions. In turn, the authors observed that functionalized leukocytes were more effective to treat cancer cells in flow compared with the soluble TRAIL or ES/ TRAIL liposomes alone. It was also found that ES/ TRAIL therapy was more effective in the induction of apoptosis when whole blood was used in comparison to COLO 205 or PC-3 cells were alone in the shearing flow. Those results suggest that the collisions among blood-cells could enhance apoptotic effects of ES/ TRAIL liposomes. The unique approach of ES/TRIAL functionalized leukocytes combined the TRAIL /ES liposomes as a targeting part and the physiological capacity of leukocytes circulate freely in the blood stream and to infiltrate tumors, enhancing the activity against CTCs.

In a similar approach, Kang and colleagues (Kang *et al.*, 2017) developed poly(lacticco-glycolic acid) (PLGA) nanoparticles coated with neutrophils membranes, to obtain a nanosized neutrophil-mimicking drug delivery system, denominated by the authors as NM-NP. This approach relies on the intrinsic cell adhesion inflammatory molecules presented by neutrophils and that could target not only CTCs but also potential metastatic niches. The neutrophils membranes were obtained from activated inflammatory neutrophils. PLGA nanoparticles used as supports were classically prepared with an emulsion/solvent protocol and NM-NP preparation was carried out by sonication of a mixture of neutrophils membrane and PLGA nanoparticles via a non-disruptive approach, preserving their biological binding



activity of ligands. The NM-NP presented an average size of 100 nm and a negative zeta potential. TEM transmission electronic microscopy investigations suggested that a single dimer neutrophil membrane layer was formed on the surface of NM-NPThe authors also encapsulated carfilzomib, a proteasome inhibitor (NM-NP-CFZ). Among the results, NM-NP exhibited a high capacity to bind to CTCs under shear stress and premetastatic endothelium model *in vitro* in shear flow. In addition, under *in vivo* conditions, NM-NP exhibited a remarkable CTCs targeting efficacy and important accumulation in premetastatic niches. Furthermore, the NM-NP-CFZ particles induced selective CTCs apoptosis in blood and prevented early metastasis and potential progression of those already established.

In another approach, Deng and colleagues (Deng *et al.*, 2015) prepared micelles of monomethyl poly(ethylene glycol)-poly(ε -caprolactone) (MPEG-PCL) loaded with doxorubicin (Dox), a well-known anti-tumor chemotherapeutic drug, by a pH-induced self-assembly method. The Dox micelles presented a size of ~27 nm, and an encapsulation yield of ~98%. When loaded with doxorubicin the IC₅₀ of Dox micelles was ~45 ng/mL, lowest than even free dox IC₅₀ (69 ng/mL). Therefore, compared with free doxorubicin, dox micelles exhibited an enhanced cytotoxicity profile, anti-tumor and anti-metastasis activity in zebrafish and mouse models. Furthermore, doxorubicin micelles appeared to target CTCs and exhibit lower side effects, characteristics highly attractive for cancer treatment.

Nowadays nanoparticles are transitioning from the multitask vehicule approach to a more rational designed drug delivery system approach. Although that the formulation of nanoparticles was conceived to overcome the different drawbacks of traditional therapy, the engineering of nanoparticles is nowadays taking more in consideration how the architecture of the nanoparticles can affect their behavior and how the nature of several molecules that are related with the metastasis could boost their activity.

Conclusion

The establishment of secondary tumors at distance sites from the primary solid tumor often determinate the last stage of cancer progression. However, the spread of cancer cells from the primary tumor can be released at the first stage of tumor growth. Thus, considering that metastasis is causing over 90% of cancer-related deaths worldwide, the detection of CTCs in patients is related to poor prognostic and low survival rates.

In the role of tumor progression, epithelial-mesenchymal transition (EMT) has been associated with the induction of invasive capacity and the formation of CTCs. It has been observed that the activation of the Snail, Slug, Twist proteins, among other molecules, could trigger EMT transition. Furthermore, the activation of EMT not exclusively is providing a fully metastasis potential but also is providing resistance to cell death, radioresistance, and chemoresistance, since the machinery of genes that is used plays a crucial role in embryonic development, wound healing and regeneration.

In CTCs seed, the margination of them is a crucial step in their migration, considering that only 0.01% of CTCs shed will form successful secondary tumors. Particle's margination can be influenced by several factors such as size, shape, volume, density. Thus, the nanoparticle's design is currently taking more in consideration the architecture's effect in margination to target CTCs and how the coupling of several molecules that are related with the metastasis could boost their activity. However, the question, "where it will be found the CTCs", remains as the challenge for nanoparticle rational design. Despite the complex environment

that nanoparticles need to face for CTCs targeting, several approaches are actively investigated as a promising highly tailored metastasis therapy.

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TRAVAUX EXPÉRIMENTAUX

CHAPITRE II

MODULATED ARCHITECTURE OF PBLG-BASED NANOPARTICLES FOR NANOMEDICINE APPLICATIONS

MODULATED ARCHITECTURE OF PBLG-BASED NANOPARTICLES FOR NANOMEDICINE APPLICATIONS

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Abstract

In recent years, considering the great advance in material science, the design of nanoparticles and in general frame nanomedicine, is transitioning from the aim to include several properties to a more rational design. This transition is mainly focused in conceive a highly custom size, shape and surface characteristics, also called as architecture, to modulate their fate in the body. Following this transition, in this work were proposed a variety of PBLG derivates under di- and tri-block form successfully synthetized by ring-opening polymerization through amine initiators. The synthetized copolymers exhibit a range of PBLG and PEG lengths. Stable and reproducible nanoparticles were successfully obtained with sizes under 100 nm and negative ζ potential. The transmission electronic microscopy (TEM) analysis revealed a variety of morphologies from rod (aspect ratio 1.3) to ellipsoidal shape (aspect ratio 2.6). According with the analysis, it can be inferred that PBLG-based nanoparticles exhibit interesting characteristics that can be modulated through polymer arrangement. Thus, PBLG-based nanoparticles could be considered as solid candidates for nanomedicine applications.

Keywords: self-assembly, nanoparticles, PBLG copolymers, margination, drug delivery systems, nanoprecipitation.

Introduction

Traditional therapy is defined by the direct administration of a small drug molecule into the body. The pharmacokinetic limitations and their non-specificity, deflect a substantial drug quantity to sites where their actions are toxic, developing important side effects. Often, to reach the target site with the effective therapeutic concentration, the dose is maintained by repetitive administration of high drug concentration (Blanco *et al.*, 2015; Coelho *et al.*, 2010; Couvreur *et al.*, 2006; Kou *et al.*, 2013), situation that exacerbates their toxic effect instead of providing an adequate therapeutic activity.

To overpass those obstacles, nanoparticles have emerged not merely to prevent drug degradation or enhance drug solubility, but also to address a precise target (Kingsley *et al.*, 2006). Dendrimers (Z. Li *et al.*, 2010), metal-polymer nanoparticles (Wen *et al.*, 2013), triblock polymeric nanoparticles (Y. Zhang *et al.*, 2005) and micelle-polymeric nanoparticles (Ko *et al.*, 2009; Navarro *et al.*, 2015; W. Zhang *et al.*, 2011) are a great example of the revolution in drug delivery systems, since the introduction of the "magic bullet" concept, theorized by Paul Ehrlich in 1891 (Cooper, 1964).

In recent years, considering the great advance in material science, the design of nanoparticles and in general frame nanomedicine, is transitioning from the aim to include several properties to a more rational design. This transition is mainly focused in conceive a highly custom size, shape and surface characteristics, also called as architecture, to modulate their fate in the body (Duan *et al.*, 2013; Jun Deng *et al.*, 2016; Toy *et al.*, 2013; Toy *et al.*, 2016).

In the literature are found several examples about how a rational design can boost nanoparticle performance once administrated. In terms of size, the nanoscale range is defined from 1 nm to 1,000 nm. However, it is found that nanoparticles within 20-200 nm could have an appropriate size for intravenous administration (Albanese *et al.*, 2012; Elsabahy *et al.*, 2012; He *et al.*, 2010; Sykes *et al.*, 2014). Nanoparticles with small size (<10 nm) or above 200 nm, could be captured by the reticuloendothelial system (RES) or filtered by lung, kidneys, liver or spleen (Alexis *et al.*, 2008; Chambers *et al.*, 2004; Toy *et al.*, 2016; Upponi *et al.*, 2014). In addition, nanoparticles within the optimal size can benefit of enhanced permeability and retention effect (EPR). First described by Matsumura and Maeda (Matsumura *et al.*, 1986), EPR phenomenon is based in the non-specific accumulation through leaky vasculature and poor lymphatic drainage in the tumor (Bazak, *et al.*, 2014). Among their results, Matsumura and Maeda also described that small molecules under 30 kDa, such as neocarzinostatin (12kDa) do not exhibit EPR effect (Maeda, 2012; Matsumura *et al.*, 1986; Matsumura, *et al.*, 1987; Upponi *et al.*, 2014). Therefore, the optimal size of nanoparticles needs to be carefully selected.

Margination is one phenomenon that is attracting the attention to considerer the importance of rational nanoparticle design, particularly in terms of shape. This phenomenon occurs when a nanoparticle that is under hydrodynamic forces in the blood flow, is deflected towards the vessel walls. In a spherical nanoparticle, the forces are homogenously distributed. Thus, the nanoparticle remains strongly captured in the blood flow, the same sequestration is observed in nanoparticles with higher sizes. In contrast, elongated nanoparticles, for which the forces are heterogeneously distributed, generate a rotational and translational movement, allowing the nanoparticle to escape from the center of the flow towards the vessel walls (Doshi *et al.*, 2010; Ponchel *et al.*, 2016; Thompson, *et al.*, 2013; Toy *et al.*, 2013). The role of

margination become more evident when the internalization of nanoparticles is necessary to reach the target. However, the elongation required to be controlled. It is found in the literature that the degree of elongation can hinder the cellular uptake. If the elongated nanoparticle is facing with the long axis the cell membrane, the obstruction is related with the greater time need it to wrap the nanoparticle. In contrast, if it is facing with the minor axis or present a spherical shape, the cellular uptake is favored (Champion, *et al.*, 2007; Verma *et al.*, 2010). In addition, nanoparticle shape could facilitate the cellular uptake through active targeting (e.g. antibodies, proteins, etc.). The elongated nanoparticles could present the ligants in a parallel orientation, allowing relaxed bindings with higher interaction points that otherwise, in the case of spherical nanoparticles due the circular angle could be tensioned (Thompson *et al.*, 2013).

Among the intrinsic characteristics of nanoparticles, the hydrophilic/hydrophobic and charge nature of the surface, influence substantially the nano-bio interface. The membrane cells with an anionic nature seems to spontaneously absorb nanoparticles with positive ζ potential or hydrophobic nature (Duan *et al.*, 2013). However, the analysis of this behavior is still complex. Nanoparticles with those characteristics also exhibit spontaneous aggregation and a high level of immunological recognition, situations that limit their effectiveness to reach the tumor site (Duan *et al.*, 2013). To avoid those situations, nanoparticles are provided with a protective layer of poly(ethylene glycol) (PEG), a highly flexible, neutral and hydrophilic polymer that avoid the opsonization and recognition by the immunological system through steric hindrance (Hu, *et al.*, 2007; Li *et al.*, 2010). Another approach is to include a surfactant into the preparation process. A commonly used surfactant is poloxamer, a triblock copolymer in ABA arrangement, that is strongly adsorbed in hydrophobic surfaces, leading a protective

layer of polyethylene oxide (Alexandridis *et al.*, 1995; Santander-Ortega, *et al.*, 2006). The advantage of this layer it not merely to enhance circulation half-life but also to functionalize the surface with antibodies, protein or molecules of interest (Kou *et al.*, 2013).

Regarding the importance of nanoparticles characteristics and their rational design, in this work, we prepared a variety of PBLG-derivates by ring-opening polymerization of γ benzyl-L-glutamate N-carboxyanhydride (BLG-NCA) through amine initiators. The set of diand tri-block amphiphilic copolymers of poly (γ -benzyl L-glutamate) (PBLG) present a variety of lengths and arrangement of PEG blocks and PBLG α -helix. The selection of copolymers is to provide a range of nanoparticle architectures once prepared by nanoprecipitation method. The aim of this work is to analyze the intrinsic characteristics of self-assembly PBLG nanoparticles, in terms of size, shape and surface features to determine their potential as drug delivery systems.

Materials and methods

Materials

Dry *N*,*N*-dimethylformamide (DMF) and dry benzylamine were purchased from Acros Organics. diethyl ether (DEE), methanol, tetrahydrofuran (THF) were purchased from Carlo Erba Reagents. deuterated chloroform (CDCl₃), trifluoroacetic acid (TFA), were purchased from Sigma-Aldrich. All the solvents, of analytical grade, were used as received unless otherwise noted. γ-benzyl-L-glutamate N-carboxyanhydride (BLG-NCA) from ISOCHEM-SNPE was used as received. α-methoxy-ω-amino-poly(ethylene glycol) (MeO-PEG-NH₂) *M*w = 5 000 gmol⁻¹, α,ω-Bis-amino poly(ethylene glycol) (H₂N-PEG-NH₂) *M*w = 10 000 gmol⁻¹, and α-methoxy-ω-carboxylic acid succinimidyl ester poly(ethylene glycol) (MeO-PEG₅-NHS)

 $Mw = 5\ 000\ \text{gmol}^{-1}$ from IRIS Biotech GMBH were dried under vacuum over argon at 30°C for 24h. Water was purified by reverse osmosis (MilliQ, Millipore).

Synthesis of PBLG derivates

The derivates of poly(γ -benzyl L-glutamate), were named following the format PBLG as the abbreviation of poly(γ -benzyl L-glutamate), and PEG as the abbreviation of poly(ethylene glycol), the subscripts indicate the molar mass in KDa. PBLG₃₅-PEG₅, PBLG₃₅-Bz, PBLG₃₅-PEG₁₀-PBLG₃₅, and PBLG₇₀-PEG₁₀-PBLG₇₀ were synthesized by Ring Opening Polymerization (ROP) of γ -benzyl-L-glutamate N-carboxyanhydride (BLG-NCA) initiated by MeO-PEG₅-NH₂, H₂N-PEG₁₀-NH₂ and benzylamine, in DMF, respectively with a slightly modified method described elsewhere (Martínez Barbosa, *et al.*, 2007).

Briefly, n milimoles of γ -benzyl-L-glutamate N-carboxyanhydride (BLG-NCA) were weighed under inert atmosphere in an argon-purged three necks round-bottomed flask. The NCA was dissolved in anhydrous DMF under argon flux and mechanical stirring at 30°C for 10 minutes. Absence of NCA auto-polymerization was confirmed by infrared spectroscopy of the BLG-NCA before addition of the initiator. The initiator solution was added with an argonpurged syringe. The reaction evolution was controlled by infrared spectroscopy. Polymers were obtained by precipitation in an excess of cold DEE. To eliminate the excess of unreacted PEG, the precipitate was filtrated and washed three times with methanol. The polymers were finally washed with DEE and dried under vacuum at room temperature for at least 12h. A second precipitation, purification and drying procedure were performed for all polymers. Synthesis of PEG₅-PBLG₇₀-PEG₅ copolymer

PEG₅-PBLG₇₀-PEG₅ was obtained by Carbodiimide crosslinker chemistry approach (Cauchois, *et al.*, 2013; de Miguel, *et al.*, 2014) from a pre-synthesized PBLG₇₀-PEG₅ copolymer. Briefly, 2 g of pre-synthesized copolymer was weighed in an argon-purged three necks round-bottomed flask and dissolved in anhydrous DMF. The solution was stirring for 10 min at room temperature with argon flux. Then a solution of PEG₅-NHS 0.02M in DMF was added with an argon-purged syringe. The reaction mixture was left for 24h. The precipitation, purification and drying procedure were performed as described above.

Characterization of PBLG derivates

FT-IR

The film of the reaction mixture was followed by FT-IR. The samples were taken directly from the reaction mixture without any further manipulation. A first step in each polymerization was performed for discard NCA auto-polymerization. The structure of the polymers was also confirmed by FT-IR measurements at room temperature on the polymer powders. The measurements were performed with a Perkin Elmer FT-IR spectrum two system.

¹H NMR

¹H NMR spectra of the PBLG derivates were recorded using a Bruker B-ACS 60 apparatus operating at 300 MHz. Samples were analyzed in CDCl₃ and 15% of TFA. For PBLG derivates containing PEG, the molar mass of the polymer could be determined by the integration of the benzyl protons belonging to the PBLG block (7.26 (br, s, 5H, Ph–) or 5.04
(br, s, 2H, –CH₂–benzyl)) and the ethylene protons of the PEG block (3.65 (br, s, 4H,–OCH₂– CH₂O–)) and taking in account the mass of the initiator.

MALDI-TOF MS

MALDI-TOF MS analyses were performed using an UltrafleXtreme mass spectrometer (Bruker Daltonics, Bremen). Acquisitions were performed in linear positive ion mode. The laser intensity was set just above the ion generation threshold to obtain peaks with the highest possible signal-to-noise (S/N) ratio without significant peak broadening. All data were processed using the program FlexAnalysis (Bruker Daltonics, Bremen). DCTB was used as the matrix for MALDI-TOF experiments and CF₃COOK was used as the cationizing agent.

Preparation and characterization of self-assembled nanoparticles

PBLG derivates nanoparticles were prepared following a modified nanoprecipitation method described elsewhere (de Miguel *et al.*, 2008). Briefly, PBLG derivates were dissolved in THF at 30°C. The solution was added dropwise to a 0.125% w/v poloxamer F68 solution or Milli-Q water under magnetic stirring. Solvents were evaporated by a standardized protocol under vacuum in the rotavapor at 40°C. The nanoparticle suspensions were filtered and stored at 4°C for further use.

The morphology of PBLG nanoparticles was analyzed by transmission electron microscopy (TEM) (JEOL-1400). Hence, 5 μ L of nanoparticles suspension were placed onto a copper grill covered with formvar-carbon film (400 mesh). After 1 min of deposition at room temperature, no-adherent nanoparticles were eliminated and the sample was stained by phosphotungstic acid (1%) for 30 s. TEM bright field imaging was performed under a 120kV accelerating voltage.

ImageJ software was used to analyzed the images. Size and zeta potential of the nanoparticles Université Paris-Saclay Université Paris Sud/ Institut Galien Paris Sud (CNRS UMR 8612), 5 rue Jean-Baptiste Clément, 92290 Châtenay-Malabry, France were determined by dynamic light scattering (DLS) using a Zetasizer 4, Malvern Instruments. Experiments and measurements were made in triplicate.

Size and shape Analysis

The nanoparticles were analyzed in terms of morphology through TEM pictures to determine the size and the shape of the resultant PBLG-nanoparticles, following the method proposed by Cauchois and colleagues (Cauchois *et al.*, 2013). As explained before, two set of PBLG-based nanoparticles were prepared. For each nanoparticle system, the dimensions were measured on the long axis (length) and the short axis (width), respectively. The analysis was performed in 100 nanoparticles from different TEM pictures. The measures were transformed in aspect ratio and equivalent volume following the equations in the figure 1. The analysis was carried out in ImageJ software.



Figure 1. Nanoparticles size and shape analysis parameters.

In addition, the number of PBLG α -helices composing each nanoparticle was calculated following the method proposed by De Miguel and colleagues with slightly modifications (de Miguel *et al.*, 2014). The calculation was obtained by the ratio between the nanoparticle volume and PBLG α -helix volume, using the equation 1 and 2. For PBLG block, the aminoacid unit have a length of 0.15 nm and a diameter of 1.6 nm (Klok *et al.*, 2000). Taking in consideration the PBLG arrangement, the segment was considered as rods. The nanoparticle volume was considered as a prolate ellipsoid (Eq. 1) or sphere (Eq. 2).

(Eq.1) Nahelix/np =
$$\frac{4(ab^2)}{3(hahelix \cdot rahelix^2)}$$

(Eq.2) Nahelix/np =
$$\frac{4(rnp^{3})}{3(hahelix \cdot rahelix^{2})}$$

Where a is the semi axis of length and b is the semi axes of width obtained by TEM measurements. rnp is the equivalent radius of the nanoparticle, hahelix and rahelix corresponds to the length and the radius of PBLG segment, respectively.

Calculation of nanoparticle amphiphilic surface

To determine the amphiphilic nature of PBLG-based nanoparticles prepared in presence or absence of poloxamer, an analysis of surface was performed (Figure 2). The calculation of the hydrophilic surface is based in the surface obtained in the interface of PBLG α -helix and PEG chain per number of α -helix chain in each nanoparticle, considering the polymer chain arrangement. This assumption is based in the core-shell nanoparticle organization, in where the PBLG will remain at the interior, indicating the limit of the hydrophobic nature. Thus, PEG chain as a shell will be projected as a hydrophilic segment.

As explained before, the PBLG chain is in α -helix and according with the literature, the α -helix diameter is 1.6 nm. Assuming a PBLG α -helix rod-type chain, the stacking of them into nanoparticles will allow to have empty space in between. Since this space cannot be occupied by other PBLG chain, the interface surface was calculated based in an equivalent square presenting the same diameter of 1.6 nm (Klok *et al.*, 2000).





Figure 2. Scheme illustration of PBLG-chain organization in PBLG-based nanoparticles and hydrophilic surface calculation method. where a is the semi axis of width and c is the nanoparticle radius obtained by TEM measurements. hahelix and rahelix corresponds to the length and the radius of PBLG segment, respectively.

To determine the of hydrophilic surface, it was first calculated the total nanoparticle's surface, using the equivalent radius (eq. 3).

(Eq. 3) Total nanoparticle's surface:
$$4\pi r^3$$
 where r: $\frac{(\sqrt[3]{Length x Width^2})}{2}$

The length and width values obtained by TEM measurements were used.

Once determined the total surface, it was calculated the hydrophilic surface following the equation 4.

(Eq. 4) hydrophilic surface: $L^2 \times N\alpha helix/np$

Where L is the diameter of PBLG α helix and N α helix/np is the quantity of PBLG α helix per nanoparticle calculated by the equation 1 and 2.

The percentage of hydrophilic surface was then calculated by the equations 5.

(Eq. 5) % hydrophilic surface: $\frac{(hydrophilic surface \times 100)}{total surface}$

The hydrophobic surface was then calculated by the difference between the total surface and the hydrophilic surface, respectively.

Results and discussion

Synthesis and characterization of PBLG derivates

In the research of biocompatible materials for the preparation of efficient drug delivery systems, synthetic polymers have attracted interest due to their enormous potential to be custom made. Random, block or graft polymer arrangement could be achieved with several functionalization degrees (Tiwari *et al.*, 2012).

Among the synthetic polypeptides is found poly (γ -benzyl L-glutamate). Also known as PBLG, it is a degradable synthetic polymer belonging to the large family of poly (α -amino acids). It has drawn attention to biomedical applications due to the presence of a degradable amide in the polymer backbone and a carboxylic acid function on the lateral chain, available after the hydrolysis of the benzyl ester. In addition, PBLG chain have a reactive amine terminal chain group. These functions give the potentiality to add additional functional groups, which would give specific properties. Therefore, PBLG can be considered as a solid candidate to compose an "ideal vector" (Anderson *et al.*, 1985; de Miguel *et al.*, 2014; Martinez Barbosa *et al.*, 2008; Martínez Barbosa *et al.*, 2007).

In this context, PBLG derivates were synthesized by γ -benzyl-L-glutamate N-carboxyanhydride (BLG-NCA) ring-opening polymerization using selectively amino-terminal compounds (Martínez Barbosa *et al.*, 2007). The amine mechanism is often used because the

primary amine is extremely more reactive than the growing chain. The initiation is faster than the propagation and therefore the degree of polymerization can be predicted by the ratio of NCA / initiator, particularly for a ratio greater than 100 (Fontaine *et al.*, 2001).

PBLG derivates were synthesized following different approaches, firstly by ringopening polymerization (ROP), using a slightly modified method described by Martinez and colleagues (Martínez Barbosa *et al.*, 2007) (Figure 3.A), and then a carbodiimide modification described by De Miguel and colleagues (de Miguel *et al.*, 2014) (figure 3.B).



Figure 3. Polymerization of γ-benzyl-L-glutamate N-carboxyanhydride leading to PBLG di-(A) and tri-block copolymers (A-B). The subscripts placed next to the brackets indicate the number of repetitive units per chain.

Polymerizations were followed by the disappearance of anhydride absorption bands at ~1855, ~1787 and ~920 cm⁻¹ corresponding to $C^5=O$, $C^2=O$ and C-O-C by FT-IR, respectively



(Figure 4). The disappearance of those bands indicated the end of the polymerization reaction.

Figure 4. FTIR spectrum of PBLG₃₅-Bz polymerization film. (I) T0min, (II) T60min.

The positions of amide absorption bands in the FT-IR spectra are used to characterize the polypeptide form. For a polypeptide in an α -helix form, the amide I band and the amide II band are located at 1656 cm⁻¹ and 1548 cm⁻¹, respectively (Fontaine *et al.*, 2001; Martínez Barbosa *et al.*, 2007; Segura-Sánchez *et al.*, 2010); and for a β -form, the amide I and amide II band are located at 1630 cm⁻¹ and 1536 cm⁻¹, respectively. Furthermore, an absorption band near to 1260 cm⁻¹ corresponding to the amide III band may be observed in the FTIR spectrum of a polypeptide in a α -helix form (Martínez Barbosa *et al.*, 2007).

For the PBLG derivates obtained in the present work, the signals corresponding to amide I and amide II bands at 1654 cm⁻¹ and 1548 cm⁻¹, also a signal located at 1260 cm⁻¹ corresponding to amide III were observed. Therefore, it can be inferred that PBLG derivates present an α -helix PBLG form (Figure 5).



Figure 5. FTIR spectrum of PBLG copolymers powders. PBLG₃₅-Bz (V), PBLG₃₅-PEG₅ (IV), PBLG₇₀-PEG₁₀PBLG₇₀ (III), PBLG₃₅-PEG₁₀PBLG₃₅ (II) and PEG₅-PBLG₇₀-PEG₅ (I).

Thus, based on the FTIR results in the figure 5, the representation of the di-block and tri-block PBLG amphiphilic copolymers are presented (figure 6). The PBLG block with a rigid hydrophobic nature and PEG block with a flexible hydrophilic nature can lead spontaneous self-association where the PBLG block will form the nanoparticle core and the PEG block will form nanoparticle shell in aqueous conditions (Martínez Barbosa et al., 2007; Segura-Sánchez et al., 2010). Due the position of PEG in the copolymer, two shell types can be expected. Brush-type were the PEG is freely at the surface and mushroom-type shell, where the two PBLG block will form a loop at the surface.

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Figure 6. Scheme of chain arrangement of PBLG derivates. The block PBLG is represented in green and the block PEG is represented in blue.

NMR and MALDI-TOF were used to determine the molar mass for each polymer. In the figure 7, the spectra of PBLG₃₅-Bz and PBLG₃₅-PEG₅ are presented. The characteristics PBLG and PEG signals were found in all the PBLG derivates obtained in this work (Supplementary Data 1), signals reported as well as in the literature (Martínez Barbosa et al., 2007; Segura-Sánchez et al., 2010).



Figure 7. ¹H NMR spectra of PBLG₃₅-Bz and PBLG₃₅-PEG₅ in CDCl3 15% TFA.

Université Paris-Saclay Université Paris Sud/ Institut Galien Paris Sud (CNRS UMR 8612), 5 rue Jean-Baptiste Clément, 92290 Châtenay-Malabry, France The molar mass of polymers containing PEG chains in the structure were determined by the ratio between the peak intensities of methylene protons of PEG chain (OCH₂CH₂) and benzyl protons of PBLG chain (COOCH₂C₆H₅) (Martínez Barbosa et al., 2007; Segura-Sánchez et al., 2010). This ratio is possible because the molar mass of PEG block is well characterized and fixed during the complete polymerization process. Due to overlapping of the peaks obtained in the NMR spectra of the polymers without PEG, the determination of the molar mass it was not possible through this technique. Thus, MALDI-TOF technique was used to overcome this problematic (Supplementary Data 2). The table 1 summarized the molar mass obtained and the synthesis conditions for each case.

			Rx					
D. I	$\overline{Dpn_t}$	NCA	[M]o	[I]0	time	Yield	<u>Mw</u>	
rorymer		(mmol)	(mol L ⁻¹)	(mol L ⁻¹)	(days)	(%)	(g mol ⁻¹)	
PBLG ₃₅ -Bz	160	13.8	0.5	0.009	3	82	45 000 ⁽²⁾	
PBLG ₃₅ -PEG ₅	160	15.2	0.025	0.009	6	80	41 000 (1)	
PEG ₅ -PBLG ₇₀ -PEG ₅	320	15.2	0. 025	0.004	1	80	109 000 ⁽¹⁾	
PBLG ₃₅ -PEG ₁₀ -PBLG ₃₅	320	15.2	0.025	0.004	7	90	72 000 ⁽¹⁾	
PBLG70-PEG10-PBLG70	640	13.6	0.025	0.002	7	90	114 000 ⁽¹⁾	
[M]o and [I]o are the initial concentration of NCA and initiator, $\overline{Dpn_t}$ indicate the theoretical degree								
of polymerization of each case. Polymers are denominated as follows: PBLG is followed by the name								
of the initiator (Bz: Benzylamine, PEG: poly(ethylene glycol)). Molar mass determined by (1) RMN,								
(2)MALDI-TOF.								

Table 1. Conditions of BLG-NCA polymerization in DMF at 30 °C.

According to the literature, it is possible to predict the chain size of PBLG block and PEG block considering the molar mass obtained. The size is calculated based in the number of repetition units present in the chain. For PBLG block, the amino acid unit have a length of 0.15 nm and a diameter of 1.6 nm (Klok *et al.*, 2000). For PEG block, the unit length is 0.28 nm in water (Oesterhelt *et al.*, 1999). Therefore, the table 2, summarize the calculated theoretical chain size for each case.

System	PBLG ₃₅ -Bz	PBLG ₃₅ -PEG ₅	PEG5-PBLG70-PEG5	PBLG ₃₅ -PEG ₁₀ -PBLG ₃₅	PBLG70-PEG10-PBLG70
Mw (g mol-1)	45 000	41 000	68 000	72 000	114 000
PEG (g mol-1)	-	5 000	10 000	10 000	10 000
PBLG Total	45 000	36 000	58 000	62 000	104 000
PBLG section	-	-	-	31 000	52 000
DP PBLG section	205	164	265	142	237
Size of PBLG (nm)	31	25	40	21	36
DP PEG Section	-	114	227	227	227
Size of PEG (nm)	-	32	64	64	64
Total Chain size (nm)	31	57	103	106	136

Table 2. Size determination of PBLG and PEG block segment of PBLG derivates.

The theoretical chain size provides valuable information to understand the organization of polymer blocks in self-assembly nanoparticles. It is interesting to remark that PEG block appeared higher than PBLG block. However, the flexibility of PEG block could allow a dynamic surface. In addition, PBLG derivates containing PEG block will exhibit an amphiphilic nature. Once self-assembled into nanoparticles, those polymers in tri-block arrangement could be expected a substantial PEG density at the surface. Further analysis is

Preparation and characterization of PBLG-based nanoparticles

In terms of polymeric nanoparticle preparation, several pathways are proposed depending of the material characteristics. Salting-out, emulsification-diffusion, solvent evaporation, are some of those methods widely used (Tiwari *et al.*, 2012). Furthermore, nanoprecipitation method is extensively used for the preparation of polymeric nanoparticles. This method allows not merely to obtain well defined nanoparticles but also incorporate a drug into them if need it (Rao *et al.*, 2011).

First developed by Fessi and colleagues in 1989, nanoprecipitation method also known as solvent displacement method is based on the interfacial disposition of a preformed, welldefined polymer, after displacement of a semi-polar solvent miscible with water from a lipophilic solution. The solvent displacement allows to form nanoparticles by spontaneous selfassembly phenomenon (Fessi *et al.*, 1989). Moreover, by controlling the characteristics of the preformed polymer, it is possible to orientate the nanoparticle architecture (Letchford *et al.*, 2007).

Thus, the preparation of novel PBLG based nanoparticles was achieved by nanoprecipitation method described elsewhere (de Miguel *et al.*, 2014; Martinez Barbosa *et al.*, 2008; Özcan *et al.*, 2011). Two set of PBLG nanoparticles were prepared, the first set was precipitated in 0.125% w/v Poloxamer solution and the second set was precipitated in Milli-Q water.

Stable and reproducible suspensions of polymeric nanoparticles were obtained for all systems in presence of poloxamer. Dynamic light scattering (DLS) measurements suggested that the nanoparticles prepared for all cases have a hydrodynamic diameter under 100 nm and a negative ζ Potential (Table 3).

System	Mean hydrodynamic diameter (nm) ^a	PDI	ζ Potential (mV) ^b
PBLG35-Bz	64 ± 2	0.090	-13 ± 0.8
PBLG35-PEG5	42 ± 1	0.099	-10 ± 0.9
PEG5-PBLG70-PEG5	47 ± 1	0.085	-11 ± 3.1
PBLG35-PEG10-PBLG35	86 ± 2	0.178	-8 ± 1.6
PBLG70-PEG10-PBLG70	65 ± 11	0.115	-10 ± 1.0

^a Measured using dynamic light scattering, n=3. ^b Measured using ZetaSizer 4, Malvern Instruments n=3. Data average of 3 batches \pm SD. PDI: polydispersity index

 Table 3. Physicochemical characterization of PBLG-based nanoparticles by nanoprecipitation

 method in 0.125 % w/v poloxamer solution.

Considering the PEG chains disposition on PBLG-based nanoparticles, some systems could present an exposed hydrophobic surface, that could be modified by poloxamer, a triblock copolymer in conformation ABA, formed by a central hydrophobic chain of polypropylene oxide (PPO) flanked by two hydrophilic chains of polyethylene oxide (PEO) (Santander-Ortega *et al.*, 2006). The PPO central part can be strongly associated into

hydrophobic nanoparticle's surface leading hydrophilic PEO chain to form a layer in the surface (Alexandridis *et al.*, 1995; De Sousa Delgado *et al.*, 2001). This effect not only stabilize nanoparticles suspension to avoid aggregation but also enhance the elastic repulsive forces to reduce nonspecific protein adsorption (De Sousa Delgado *et al.*, 2001). In addition, it has been also reported that using poloxamer in the formulation process, can reduce the size of the resulting nanoparticle (Yan *et al.*, 2010).

Thus, the second set was prepared in absence of poloxamer. Stable and reproducible aqueous suspensions were obtained for all systems beside PBLG₃₅-Bz system (Table 4). In our case, the strong hydrophobic surface lead to spontaneous aggregation. It is found for the systems containing PEG block, increased sizes in comparison to those systems prepared in presence of poloxamer. This behavior was also described in the literature (Yan *et al.*, 2010). However, the average size for all systems remained under 100 nm.

In terms of surface's charge, it is found higher negative ζ potential, for all systems. This increment is caused by the lack of the neural protection layer provided by poloxamer. Although, for the PBLG₃₅-PEG₁₀-PBLG₃₅ system, the ζ potential remains similar regardless the presence of poloxamer. This could be explained by PEG disposition that remains protecting the hydrophilic compact core.

System	Mean hydrodynamic diameter (nm) ^a	PDI	ζ Potential (mV) ^b
PBLG35-PEG5	57 ± 10	0.135	-13 ± 1.1
PEG5-PBLG70-PEG5	98 ± 19	0.129	-19 ± 0.9
PBLG35-PEG10-PBLG35	82 ±2	0.100	-9 ± 3.8
PBLG70-PEG10-PBLG70	73 ± 1	0.092	-17 ± 0.5

^a Measured using dynamic light scattering, n=3. ^b Measured using ZetaSizer 4, Malvern Instruments n=3. Data are the average of 3 batches \pm SD. PDI: polydispersity index

Table 4. Physicochemical characterization of PBLG-based nanoparticles by nanoprecipitation

 method in aqueous suspension.

Size and shape analysis

A supplementary size analysis was performed by the measure of 100 nanoparticles by the mean of the maximal length and the maximal width of each nanoparticle prepared in presence of poloxamer (Table 5). The sizes founded by TEM are near to those measured by DLS, except for PBLG₃₅-PEG₁₀-PBLG₃₅ where the compact PBLG core could allow a substantial PEG neutral density in the surface.

System	Mean size	Aspect ratio	Equivalent diameter
	(nm)	Г	
PBLG35-Bz	46 ± 8	1.2 ± 0.2	41 ± 7
PBLG35-PEG5	34 ± 3	1.6 ± 0.3	25 ± 3
PEG5-PBLG70-PEG5	49 ± 4	2.5 ± 0.4	27 ± 4
PBLG35-PEG10-PBLG35	34±4	1.3 ± 0.2	29 ± 4
PBLG70-PEG10-PBLG70	57 ± 6	2.3 ± 0.5	33 ± 6

Data are the average of 3 batches \pm SD.

Table 5. Size determination from TEM measurement as the mean between the maximal length and the maximal width of each nanoparticle prepared by nanoprecipitation method in 0.125% w/v of poloxamer solution, using negative stain (n=100).

The analysis performed by TEM also revealed the aspect ratio and the morphology of the nanoparticles prepared. The aspect ratio is common used to analyze the shape factor on nanoparticles. This parameter quantifies how near the shape of a particle approach the perfect sphere since the dimension in a sphere is equal independently of the point selected toward the center. It is calculated by the division of the maximal length and the maximal width measured (Wadell, 1935). The TEM micrographs obtained for each system are given in Figure 8.



Figure 8. TEM micrographs at 120 kV obtained from the following PBLG derivates prepared by nanoprecipitation method in 0.125% w/v poloxamer solution, using negative stain. (a) PBLG₃₅-Bz, (b) PBLG₃₅-PEG₅, (c) PEG₅-PBLG₇₀-PEG₅, (d) PBLG₃₅-PEG₁₀-PBLG₃₅, (e) PBLG₇₀-PEG₁₀-PBLG₇₀.

The determination of aspect ratio allows us to divide the systems into two main groups. The semi-spherical group is composed by PBLG₃₅-Bz (Γ =1.2) and PBLG₃₅-PEG₁₀-PBLG₃₅ (Γ =1.3). In this group, their morphology appeared as small compact spheres.

The second group is composed by PBLG₃₅-PEG₅ (Γ =1.6), PEG₅-PBLG₇₀-PEG₅ (Γ =2.5) and PBLG₇₀-PEG₁₀-PBLG₇₀ (Γ =2.5). Their aspect ratio indicates that these systems present an elongated morphology. For the systems PEG₅-PBLG₇₀-PEG₅ and PBLG₇₀-PEG₁₀-PBLG₇₀, effectively, their morphology appeared as ellipsoidal nanoparticles. However, for PBLG₃₅-PEG₅ system, as it is suggested by its aspect ratio of 1.6, is likely a rod nanoparticle.

The difference between PBLG₃₅-PEG₁₀-PBLG₃₅ system (morphology roughly spherical) and PBLG₇₀-PEG₁₀-PBLG₇₀ system (ellipsoidal morphology) can be explained by the auto-assembled phenomena of the PBLG's core. The small PBLG chain in the system PBLG₃₅-PEG₁₀-PBLG₃₅ can form a compact core. In contrast, the long PBLG chain of PBLG₇₀-PEG₁₀-PBLG₇₀ guide the stacking along the longitudinal axis of the PBLG chain.

Continuing with the analysis of the nanoparticles prepared in presence of poloxamer, the number of PBLG α -helix was calculated based in the volume of a prolate ellipsoid obtained by TEM and the volume of PBLG segment. It is found that PBLG₃₅-Bz system is formed by 581 α -helix. This result is in concordance with the spherical morphology and the size of ~46nm, that allows a highly compact nanoparticle. For PBLG₃₅-PEG₁₀-PBLG₃₅ system, also with spherical morphology but with a core/shell conformation, it is found that 289 α -helix are forming the core. It is important to recall that tri-block present one chain of PEG per 2 α -helix. Thus, PBLG₃₅-PEG₁₀-PBLG₃₅ system is formed by 145 polymer chains. Also, by TEM in where is possible to observe a well-defined core, the size is ~34nm in contrast with the size by DLS, in where it is possible to measure the hydrodynamic radius, a size of ~86nm. Therefore, both techniques complement the nanoparticle characterization.

Following with the tri-block systems, PBLG₇₀-PEG₁₀-PBLG₇₀ system, that also share the same mushroom-type PEG conformation, is formed by 258 α -helix. Therefore, the resultant quantity of PEG chains is 129. If we compare the sizes measured by TEM and DLS, the difference it is not as evident as for PBLG₃₅-PEG₁₀-PBLG₃₅ system. This could be an effect of PEG distribution. It can be considered, regarding the morphology, that the PEG is approximately equally dispersed at the extremities of the long axis. Therefore, it is not as distributed as their homologous PBLG₃₅-PEG₁₀-PBLG₃₅ system.

Furthermore, PEG₅-PBLG₇₀-PEG₅ system is composed by 123 α-helix, resulting in 246 PEG chains. The higher quantity of PEG chains above all the systems. However, as observed in PBLG₇₀-PEG₁₀-PBLG₇₀ system, non-substantial difference is found among the size measured by DLS and by TEM. This could be due to the distribution of PEG chains in the extremities of the long axis and their great flexibility. PBLG₃₅-PEG₅ system also presenting hairy-type PEG conformation, non-substantial difference is observed by both measurements. PBLG₃₅-PEG₅ system is composed by 165 α -helix, equal quantity of PEG chains is expected. Regarding the morphology of those systems, the higher quantity of PEG and the relatively long PBLG segment, orient to PEG₅-PBLG₇₀-PEG₅ system to form a thin elongated morphology in comparison to the rod morphology of PBLG₃₅-PEG₅ system.

For the systems prepared in absence of poloxamer, a supplementary size analysis was performed under the same conditions (Table 6). It is found a substantial difference among the measures obtained by TEM and DLS. However, the size increment observed in DLS is also observed by TEM. Therefore, it appears proportional. For example, PEG₅-PBLG₇₀-PEG₅ system, with a DLS size of ~98nm, by TEM is ~41nm. For the systems, PBLG₃₅-PEG₁₀-PBLG₇₀-PEG₅ and PBLG₇₀-PEG₁₀-PBLG₇₀, with sizes by DLS of ~82nm and ~73nm, by TEM presents ~63nm and ~68nm, respectively.

System	Mean size	Aspect ratio	Equivalent diameter
	(nm)	Г	
PBLG ₃₅ -PEG ₅	45 ± 8	1.2 ± 0.1	40 ± 7
PEG ₅ -PBLG ₇₀ -PEG ₅	41 ± 7	1.5 ± 0.4	33 ± 4
PBLG35-PEG10-PBLG35	63 ± 11	1.4 ± 0.4	47 ± 7
PBLG70-PEG10-PBLG70	68 ± 9	1.5 ± 0.4	53 ± 10

Data are the average of 3 batches \pm SD.

Table 6. Size determination from TEM measurements as the mean between the maximal length and the maximal width of each nanoparticle prepared by nanoprecipitation method in Milli-Q water (n=100).

The analysis performed by TEM also revealed the aspect ratio and the morphology of the nanoparticles prepared. The TEM micrographs obtained for each system are given in Figure 9.



Figure 9. TEM micrographs at 120 kV obtained from the following PBLG derivates prepared by nanoprecipitation method in MilliQ-water, using negative stain. (b) PBLG₃₅-PEG₅, (c) PEG₅-PBLG₇₀-PEG₅, (d) PBLG₃₅-PEG₁₀-PBLG₃₅, (e) PBLG₇₀-PEG₁₀-PBLG₇₀.

Regarding the TEM micrographs, the second set prepared in Milli-Q water, preserve similar morphology as observed in the first set (systems prepared in presence of poloxamer). However, it is possible to observe in those systems with ellipsoidal shape, an increment in proportion in the central part. This increment is translated into the resultant aspect ratio, in which the tendency is to the spherical value.

In detail, PBLG₃₅-PEG₅ system with a rod morphology presents an aspect ratio of 1.2, lower than the one in poloxamer (Γ =1.6). In terms of α -helix, the increased size is a result of the 685 α -helix composing the nanoparticle. Despite the higher number of α -helix, in comparison with its homologous in poloxamer (165 α -helix), a slightly difference is observed between DLS and TEM measurements. In the case of PEG₅-PBLG₇₀-PEG₅ system, the difference by TEM and DLS is clear. This system is formed by 242 α -helix with a total of 484 PEG chains distributed along both length axis. Regarding the morphology of the system, the nanoparticle central part is substantially higher, passing from a thin elongated nanoparticle with an aspect ratio of 2.6 when prepared in poloxamer, to an ellipsoidal shape with an aspect ratio of 1.5.

Furthermore, PBLG₃₅-PEG₁₀-PBLG₃₅ system and PBLG₇₀-PEG₁₀-PBLG₇₀ system preserved the spherical and ellipsoidal shape, as observed before. However, the higher central part observed in PBLG₇₀-PEG₁₀-PBLG₇₀ system, drop the aspect ratio from 2.4 in presence of poloxamer to 1.5 in presence of water. For PBLG₃₅-PEG₁₀-PBLG₃₅ system, the aspect ratio remains slightly similar. In terms of quantity of α -helix chains, it is found for PBLG₃₅-PEG₁₀-PBLG₃₅ system, 1301 α -helix forming the core and 651 PEG chains forming the shell. Quantity remarkably above from its homologous in presence of poloxamer (~289 α -helix). A comparable result it is found for PBLG₇₀-PEG₁₀-PBLG₇₀ system, in which is found 1099 α - helix chains in the core and therefore, 550 PEG chains forming the shell, remarkably higher in comparison with its homologous in poloxamer (~258 α -helix).

Through the analysis, the characteristics of PBLG-based nanoparticles could be modulated by the position of PEG chain and the molar mass of the polymer chain. As reported also in the literature, the aspect ratio is chain-length dependent. Cauchois and colleages observed that it is possible to increase the elongation of polymeric PBLG nanoparticles by increasing the polymer molar mass (Cauchois *et al.*, 2013). In addition, the effect of poloxamer on PBLG-based nanoparticles it is really clear. The presence of poloxamer is not merely stabilizing the surface to avoid aggregation, but also, is limiting the size as it was obtained smaller nanoparticles when used. In the figure 10, it is presented a scheme illustration of PBLG-based in this work as nanoparticles in presence or absence of poloxamer. The distinctive characteristics in each nanoparticle made them independent systems that could, therefore, interact in diverse ways to the environment/cells once administrated.





Figure 10. Scheme illustration of PBLG-based nanoparticles morphology in presence/absence of poloxamer.

Calculation of nanoparticle amphiphilic surface

Considering the information obtained, it was possible to calculate the amphiphilic nature of PBLG-based nanoparticles. The impact of hydrophobic/hydrophilic nanoparticle surface on nano-bio interface interaction is well-known. Protein absorption, cell interaction/up-take, immune-recognition, aggregation, etc., are some excellent examples that can be modulated by the amphiphilic nanoparticle's nature (Cedervall *et al.*, 2007; Nel *et al.*, 2009). Therefore, this determination will provide remarkable information not only about the nanoparticle organization but also to understand their nano-bio interface profile.

Table 7 summarizes the surface analysis for PBLG-based nanoparticles prepared in presence of poloxamer. PBLG₃₅-Bz system prepared in presence of poloxamer, exhibits a

total surface of 5272 nm². Since this system lack PEG chain, the calculated surface is completely hydrophobic. However, as explained before, the poloxamer may be strongly attached in the surface creating a neutral protective layer.

Aspect ratio	System	Equivalent Diameter nm	Nahelix/np	Total Surface nm ²	Hydrophobic surface nm ²	Hydrophilic surface nm ²
1.2	PBLG ₃₅ -Bz	41	581	5272	5272	-
1.3	PBLG35-PEG10-PBLG35	25	165	1964	1542	422
1.6	PBLG ₃₅ -PEG ₅	27	123	2218	1588	630
2.6	PEG ₅ -PBLG ₇₀ -PEG ₅	29	289	2579	1839	740
2.4	PBLG70-PEG10-PBLG70	33	258	3378	2057	1320

Table 7. Surface analysis of PBLG-based nanoparticles prepared in 0.125% poloxamer solution. Equivalent diameter calculated with the values obtained by TEM measurement using negative stain (n=100).

Following with the analysis, PBLG₃₅-PEG₅ system prepared in poloxamer, presents a total surface of ~1964 nm² with a hydrophilic surface of ~422 nm², equivalent to 21% of the total surface. Therefore, it can be inferred considering the morphology, that the nanoparticle presents a well dispersed PEG chain distribution. Furthermore, the poloxamer may also be absorbed at the 79% hydrophobic surface. In contrast, PBLG₃₅-PEG₅ system prepared in absence of poloxamer exhibit a total surface of ~5078 nm² with a hydrophilic surface of ~1754 nm², corresponding to 35% of the total surface. Regarding the brush-type PEG organization and the quantity of PEG chains (~685 chains) in the nanoparticle, it can be considered that the remaining 65% hydrophobic surface is maintaining as a well-protected core. In the table 8, the

Aspect ratio	System	Equivalent diameter nm	Nahelix/np	Total Surface nm ²	Hydrophobic surface nm ²	Hydrophilic surface nm ²
1.2	PBLG ₃₅ -PEG ₅	40	685	5078	1754	3324
1.4	PEG5-PBLG70-PEG5	33	242	3482	1239	2243
1.6	PBLG35-PEG10-PBLG35	47	1301	7032	6661	372
1.5	PBLG70-PEG10-PBLG70	53	1099	3825	5626	3251

summary of surface analysis of PBLG-based nanoparticles prepared in Milli-Q water is shown.

Table 8. Surface analysis of PBLG-based nanoparticles prepared in Milli-Q water. Equivalent diameter calculated with the values obtained by TEM measurement using negative stain (n=100).

Concerning the tri-block systems, it is observed in PBLG₃₅-PEG₁₀-PBLG₃₅ system prepared in poloxamer, a total surface of ~2579 nm² with a hydrophilic surface of ~740 nm², corresponding to 29 % of the total surface. This result is in concordance with the morphology that evidence the organization of the two blocks towards the center, allowing more hydrophobic surface. However, its spherical compact core is protected by the loop of its long PEG chain (~63 nm). In addition, when the system is prepared in water, the total surface is ~7032 nm² with a hydrophilic surface of ~3330 nm², representing 47% of the total surface. As observed before, no substantial difference was found, this behavior indicated a strong oriented selfassemblage process that is preserved despite the increment in sizes.

Continuing with the mushroom-type PEG conformation, $PBLG_{70}$ -PEG₁₀-PBLG₇₀ system prepared in presence of poloxamer presents a total surface of ~1320 nm² with a hydrophilic surface of 1320 nm², representing 39% of the total surface. Regarding the

percentage calculated and the morphology of the system, it could be inferred that the PEG chains are homogenously dispersed along both terminal part of the long axis. Thus, it is the central part which will expose the hydrophobic surface. In the case of PBLG₇₀-PEG₁₀-PBLG₇₀ system prepared in water, the total surface is increased to ~8877 nm² with a hydrophilic surface of ~5626 nm², representing approximately the 63% of the total surface. This is directly related with the increment in size and consequently in the number of α -helix per nanoparticle. The system is formed by 1099 α -helix, resulting in 549 PEG chains. Even though the shape is preserved, the central part is bigger, in comparison when prepared in poloxamer.

The PEG₅-PBLG₇₀-PEG₅ system prepared in poloxamer, presents a total surface of 2218 nm² with a hydrophilic surface of 630 nm², representing the 28% of the total surface. This system was conceived to have a remarkable amphiphilic nature, considering that each α -helix is formed by two PEG chains. As observed in the PBLG₇₀-PEG₁₀-PBLG₇₀ system, the hydrophilic surface may be focused at the long axis extremities, leaving a hydrophobic gap in the center. This theory is supported by the shape observed by TEM and the quantity of α -helix; in which it is observed a thin elongated nanoparticle formed by 123 α -helix. However, in presence of poloxamer, a complete hydrophilic/neutral layer could be expected. In contrast, this system prepared in water exhibit a total surface of 3482 nm² with 1239 nm² of hydrophilic surface correlates the size measured by TEM. Regarding the morphology, it is observed an increment in the central part. However, the length is preserved similar to the system prepared in presence of poloxamer (~34nm). Therefore, it can be inferred that the nanoparticle organization remains similar but higher stacking is expected. Those results suggest that PEG₅-PBLG₇₀-PEG₅ system



could present a highly dense PEG layer located in the extremities. Considering the flexibility of PEG chain, the hydrophobic surface at the center part could be protected by this layer.

Conclusion

The rational design of nanoparticles has the aim of fabricate highly defined nanoparticles to overpass the biological barriers and reach the target efficiently. The selection of nanoparticle architecture is based often in the size, surface, shape and target localization. Therefore, it is a crucial step in the formulation of novel nanoparticles, the determination of their intrinsic characteristics to fully exploit their potential.

In this work were successfully synthetized a variety of PBLG derivates in di- and triblock conformation by ring-opening polymerization of γ -benzyl-L-glutamate Ncarboxyanhydride (BLG-NCA) in presence of various amino-based initiators. The synthetized copolymers exhibit a range of PBLG and PEG lengths, that were calculated based on the molar mass obtained and the intrinsic chain sizes. The α -helix conformation of PBLG chain with a hydrophobic nature, combined with hydrophilic PEG chain allowed to obtain self-assemble amphiphilic nanoparticles with hairy-type and mushroom-type surface PEG conformation by nanoprecipitation method.

Two set of PBLG-based nanoparticles were prepared, one in presence of poloxamer and the second in absence of poloxamer, a triblock copolymer that can be strongly absorbed in hydrophobic surfaces. Stable and reproducible nanoparticles prepared in presence of poloxamer were successfully obtained with sizes under 100 nm and negative ζ potential. In addition, PBLG-based nanoparticles prepared in absence of poloxamer exhibit sizes under 100 nm with higher negative ζ potential. The increment in the negative ζ potential is the reflex of the lack poloxamer absorption in the exposed hydrophobic surface. The TEM analysis revealed a variety of morphologies from rod (aspect ratio 1.3) to ellipsoidal shape (aspect ratio 2.6). It was also found that the morphology is preserved despite the use of poloxamer during the preparation process. Through the surface analysis, it was determined that PBLG-based nanoparticles exhibit an amphiphilic nature, particularly in ellipsoidal tri-block nanoparticles.

Therefore, considering the results obtained, it can be inferred that PBLG-based nanoparticles exhibit interesting characteristics that can be modulated through polymer arrangement. Thus, PBLG-based nanoparticles could be considered as solid candidates for nanomedicine applications.

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CHAPITRE III

RATIONAL DESIGN OF POLY(γ-BENZYL L-GLUTAMATE) NANOPARTICLES FOR NANOMEDICINE APPLICATIONS IN CANCER TREATMENT

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RATIONAL DESIGNED POLY(γ-BENZYL L-GLUTAMATE) NANOPARTICLES FOR NANOMEDICINE APPLICATIONS IN CANCER TREATMENT

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Abstract

Cancer is one of the leading causes of morbidity and mortality worldwide with approximately 14 million new cases and 8.2 million cancer related deaths in 2014, reported by the World Health Organization, number that is increasing through time. The most concerning aspect is that nearly 90% of those cancer deaths are related directly to tumor spread in distant organs, referred to as metastasis. A key factor in metastasis process is the production of circulating tumor cells (CTCs), highly aggressive cells that are released to bloodstream even in early steps of tumor progression. Considering that CTCs could be treated in circulation, it is clear the necessity to develop highly designed nanomedicines to efficiently target CTCs. In the present work, a mini-library of di- and tri-block amphiphilic copolymers is proposed to be candidates to CTCs treatment. The PBLG derivates were synthetized by introducing varying lengths and configurations of PEG blocks. Each modification of the copolymers provided different architectures, characterized by a variety of sizes, shapes and conformations of PEG chains at the surface of the resulting nanoparticles. The present study suggests that these morphological modifications could impact nanoparticles interactions with biological components and could therefore be interesting for modulating their disposition in the body.

Keywords: nanoparticles, PBLG, copolymer, amphiphilic, therapy

Introduction

Nanomedicines have been proposed to treat a wide-range of diseases as drug delivery systems (DDS). Since decades, researchers have been developing several materials and strategies to build highly specialized DDS (Couvreur *et al.*, 2006; Nicolas *et al.*, 2013). To overpass the biological barriers, long-time circulation, biocompatibility, submicron sizes, furtiveness, smart addressing, are some of the characteristics of this type of devices (Jain, 2008). Many drugs, which exhibit suboptimal characteristics when dosed by conventional delivery systems, can benefit from these characteristics, which are expected to modify/increase their efficacy as well as their safety (benefit/risk ratio) (Martínez Barbosa *et al.*, 2007; Coelho *et al.*, 2010).

Considering the vast diversity of materials and their resulting configuration when selfassembly, a critical step in the design of highly specialized DDS is to achieve a balance between their surface, size and shape characteristics at the final DDS architecture (Kou *et al.*, 2013). The optimal balance of those parameters can enhance the efficacy of DDS but a nonappropriated architecture selection can lead to have poor biodistribution, cytotoxicity, spontaneous clearance by the body or immune recognition/elimination (Alexis *et al.*, 2008). This balance is also founded in the nature, where viruses, bacteria, parasites and other significant biological structure have evolved into defined architectures to achieve target interactions and functions (Albanese *et al.*, 2012).

After DDS administration, a phenomenon that is linked to the morphology of the particle is the marginalization. This phenomenon occurs when the DDS exposed in a fluid is influenced in their motion by surrounded forces (e.g. electrostatic interactions, van der Waals

interactions, steric repulsive interactions, buoyancy, among others) towards the wall of the blood vessels (Carboni *et al.*, 2014). In the case of spherical particles, the homogeneous distribution of the force conducts the particle through the center of the vessel. Meanwhile in the case of non-spherical particles, the heterogeneous distribution of the force, torques and deviations due the orientation angle conducts the particle to different directions in the vessel, allowing them to escape from the flow and enhance internalization opportunities (Gentile *et al.*; Toy, 2014). The margination phenomenon it is not only affected by the nanoparticle shape, it is also affected by the size. Experimental evidence among the literature shows that small nanoparticles have higher diffusion coefficient allowing them to move with relative freedom along the vessel. On the contrary, bigger nanoparticles are more kidnapped by the forces, turning difficult to them to escape from the flow (Thompson *et al.*, 2013; Toy *et al.*, 2013).

The effect of the shape is not only at the level of the marginalization phenomenon; it is also a crucial parameter at nanoparticle-cell interaction and toxicity (Sharma *et al.*, 2010; Greish *et al.*, 2012; Mathaes *et al.*, 2015; Grabowski *et al.*, 2016). It is found in the literature that spherical or small nanoparticles are more likely internalized than non-spherical nanoparticles, due the greater membrane wrapping time required for elongated nanoparticles (Verma *et al.*, 2010; Toy *et al.*, 2013; Firdessa *et al.*, 2014).

The membrane wrapping time is also governed by the surface of DDS architecture when ligands are involved (Doshi *et al.*, 2010). In elongated nanoparticles, the ligands are exposed with a horizontal orientation facing the cell membrane. This orientation concedes not only more approachable sites but also less tensioned unions. Contrary to the elongated nanoparticles, the curvature of spherical nanoparticles increases the distance among the union causing high tension on them. Weak binding interactions can be a result of that tension and consequently

poor binding avidity. Even though, the size and the shape are important parameters, it is the chemical functionalities of the surface and their arrangement that dictate nanoparticle-cell interactions (Verma e Stellacci, 2010; Albanese *et al.*, 2012).

The analysis of DDS architecture effect is nowadays a tendency in nanomedicine. Elucidate the effect of size, shape and surface in DDS design will increase the ability to engineer new generations of nanoparticles capable to modulate their fate in the body. Although we can find in the literature several studies of DDS architecture, this part is still growing.

In the present work, we prepared a set of di- and tri-block amphiphilic copolymers of Poly (γ -benzyl L-glutamate) (PBLG) by introducing varying lengths and configurations of PEG blocks. Each modification of the copolymers provided different architectures, characterized by a variety of sizes, shapes and conformations of PEG chains at the surface of the resulting nanoparticles. The present study suggests that these morphological modifications could impact nanoparticles interactions with biological components and could therefore be interesting for modulating their disposition in the body.

Materials and methods

Materials

Dry *N*,*N*-dimethylformamide (DMF) and dry benzylamine were purchased from Acros Organics. diethyl ether (DEE), methanol, tetrahydrofuran (THF) were purchased from Carlo Erba Reagents. deuterated chloroform (CDCl₃), trifluoroacetic acid (TFA), rhodamine B and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (MTT), Dulbecco's phosphate buffered saline (PBS), Dulbecco's phosphate buffered saline (PBS),

Dulbecco's Modified Eagle's Medium - high glucose, RPMI-1640 Medium, penicillin (100 Université Paris-Saclay Université Paris Sud/ Institut Galien Paris Sud (CNRS UMR 8612), 5 rue Jean-Baptiste Clément, 92290 Châtenay-Malabry, France U/ml), streptomycin (100 µg/ml), trypsin and paraformaldehyde (PFA), were purchased from Sigma-Aldrich. All the solvents, of analytical grade, were used as received unless otherwise noted. y-benzyl-L-glutamate N-carboxyanhydride (BLG-NCA) from ISOCHEM-SNPE was used as received. α -methoxy- ω -amino-poly(ethylene glycol) (MeO-PEG₅-NH₂) Mw = 5000gmol⁻¹ and α, ω -Bis-amino poly(ethylene glycol) (H₂N-PEG₁₀-NH₂) $Mw = 10\ 000\ \text{gmol}^{-1}$, α methoxy- ω -carboxylic acid succinimidyl ester poly(ethylene glycol) (MeO-PEG₅-NHS) Mw =5000 gmol⁻¹ from IRIS Biotech GMBH were dried under vacuum over argon at 30°C for 24h. Agarose, tricine, tris base (Sigma 7-9[®]), sodium chloride and bromophenol blue were purchased from Sigma (Saint-Quentin-Fallavier, France). Human serum was prepared from plasma provided by Etablissement Français du Sang (EFS) (Rungis, France). Polyclonal antihuman C3 antibody raised in goat was purchased from Fitzgerald antibodies (Acton, USA). Coomassie brilliant blue R-250, calcium lactate and glacial acetic acid were supplied by Thermo Fisher Scientific (Villebon-sur-Yvette, France). Gel-Fix[™] for agarose (265x150 mm) was purchased from Serva Electrophoresis (Heidelberg, Germany). Cobra Venom Factor was purchased from Quidel (Athens, USA). Water was purified by reverse osmosis (MilliQ, Millipore).

Methods

Synthesis of PBLG derivatives

The derivates of poly(γ -benzyl L-glutamate), were named following the format PBLG as the abbreviation of poly(γ -benzyl L-glutamate), and PEG as the abbreviation of poly(ethylene glycol). In each compound, the subscripts indicate the molar mass in KDa. PBLG₃₅-PEG₅, PBLG₃₅-Bz, PBLG₃₅-PEG₁₀-PBLG₃₅ and PBLG₇₀-PEG₁₀-PBLG₇₀ were

synthesized by Ring Opening Polymerization (ROP) of γ -benzyl-L-glutamate N-carboxyanhydride (BLG-NCA) initiated by MeO-PEG₅-NH₂, H₂N-PEG₁₀-NH₂ and benzylamine, in DMF, respectively with a slightly modified method described elsewhere (Supplementary Data 1) (Martínez Barbosa *et al.*, 2007).

Briefly, n millimoles of BLG-NCA were weighed under inert atmosphere in an argonpurged three necks round-bottomed flask. The NCA was dissolved in anhydrous DMF under argon flux and mechanical stirring at 30°C during 10 minutes. Absence of NCA autopolymerization was confirmed by infrared spectroscopy of the NCA before addition of the initiator. The initiator solution was added with an argon-purged syringe. The reaction evolution was controlled by infrared spectroscopy. Polymers were obtained by precipitation in an excess of cold DEE. The precipitate was filtrated and washed three times with methanol, to eliminate the excess of unreacted PEG. The polymers were finally washed with DEE and dried under vacuum at room temperature for at least 12h. A second precipitation, purification and drying procedure were performed for all polymers.

Synthesis of PEG₅-PBLG₇₀-PEG₅ copolymer

PEG₅-PBLG₇₀-PEG₅ were obtained by carbodiimide crosslinker chemistry approach (Cauchois *et al.*, 2013; De Miguel *et al.*, 2014). PEG₅-PBLG₇₀-PEG₅ was obtained from a presynthesized PBLG₇₀-PEG₅ copolymer (Supplementary Data 2).

Briefly, 2 g of pre-synthesized copolymer was weighed in an argon-purged three necks round-bottomed flask and dissolved in anhydrous DMF. The solution was stirring for 10 min at room temperature with argon flux. Then a solution of PEG₅-NHS 0.02M in DMF was added



with an argon-purged syringe. The reaction mixture was left for 24h. The precipitation, purification and drying procedure were performed as described above.

Synthesis of PBLG₃₅-Cyanine 3

PBLG₃₅-Cyanine 3 was obtained by a carbodiimide crosslinker chemistry approach, following a slightly modified method (Supplementary Data 3) (Segura-Sánchez *et al.*, 2010; De Miguel *et al.*, 2014). We used a pre-synthesized PBLG₃₅-Bz obtained by ROP as described above. Briefly, 2 g of PBLG₃₅-Bz and 0.02g of EDC were weighed in an argon-purged three necks round-bottomed flask and dissolved in DMF. The solution was stirring for 10 minutes at room temperature with argon flux. Then, a solution of Cyanine 3 0.05M in anhydrous DMF was added with an argon-purged syringe. The reaction mixture was left for 24h in darkness. The precipitation, purification and drying procedure were performed as described above. The polymer was washed several times with methanol to eliminate the excess of unreacted Cyanine 3.

Characterization of PBLG derivatives

FT-IR

The film of the reaction mixture was followed by FT-IR. The samples were taken directly from the reaction mixture without any further manipulation. A first step in each polymerization was performed for discard NCA auto-polymerization. The structure of the polymers was also confirmed by FT-IR measurements at room temperature on the polymer powders. The measurements were performed with a Perkin Elmer FT-IR spectrum two system.

¹H NMR

¹H NMR spectra of the PBLG derivatives were recorded using a Bruker B-ACS 60 apparatus; operating at 300 MHz. Samples were analyzed in CDCl₃ and 15% of TFA. For PBLG derivatives containing PEG, the molar mass of the polymer could be determined by the integration of the benzyl protons belonging to the PBLG block (7.26 (br, s, 5H, Ph–) or 5.04 (br, s, 2H, $-CH_2$ –benzyl)) and the ethylene protons of the PEG block (3.65 (br, s, 4H, $-OCH_2$ – CH₂O–)) and taking in account the mass of the initiator.

MALDI-TOF MS

MALDI-TOF MS analyses were performed using an UltrafleXtreme mass spectrometer (Bruker Daltonics, Bremen). Acquisitions were performed in linear positive ion mode. The laser intensity was set just above the ion generation threshold to obtain peaks with the highest possible signal-to-noise (S/N) ratio without significant peak broadening. All data were processed using the program FlexAnalysis (Bruker Daltonics, Bremen). DCTB was used as the matrix for MALDI-TOF experiments and CF₃COOK was used as the cationizing agent.

Preparation and characterization of self-assembled nanoparticles

PBLG derivatives nanoparticles were prepared following a modified nanoprecipitation method described elsewhere (Martínez Barbosa *et al.*, 2007; De Miguel *et al.*, 2014). Briefly, a single or mixtures of PBLG derivatives were dissolved in THF at 30°C. The solution was added dropwise to a 0.125% w/v poloxamer F68 solution or Milli-Q water, under magnetic stirring. Solvents were evaporated by a standardized protocol under vacuum in the rotavapor at 40°C. The nanoparticle suspensions were filtered and stored at 4°C for further use.

The morphology of PBLG nanoparticles was analyzed by transmission electron microscopy (TEM) (JEOL-1400). For that, 5 μ L of nanoparticles suspension were placed onto a copper grill covered with formvar-carbon film (400 mesh). After 1 min of deposition at room temperature, no-adherent nanoparticles were eliminated, and the sample was stained by phosphotungstic acid (1%) for 30 s. TEM bright field imaging was performed under a 120kV accelerating voltage. ImageJ software was used for analyzed the images. Size and zeta potential of the nanoparticles were determined by dynamic light scattering (DLS) using a Zetasizer 4, Malvern Instruments. Experiments and measurements were made in triplicate.

Biocompatibility of PBLG nanoparticles

Multi-crossed electrophoresis (µC-IE) for the determination of protein C3 activation

The method of multi-crossed 2D immunoelectrophoresis described by Coty and colleagues (Passirani *et al.*, 1998; Coty *et al.*, 2016) was performed for the analysis of complement C3 activation. Briefly, AMB BSA samples (50μ L) was incubated with Human serum (25 μ L) in Veronal Buffer Saline (VBS2+) (25 μ L) prepared as described by (Kazatchkine, 1985). Samples were incubated for 1 h at 37°C. Then, they were immediately cooled to 4°C in an ice bath for immediate analysis by μ C-IE.

Agarose gel 1% containing anti-C3 antibody was prepared in Tricine buffer (Calcium Lactate 1 mM, TRIS 63 mM, Tricine 27 mM, in milliQ® water, pH 8.6) and then casted on a Gel-FixTM film. Bands were cutted and then filled with 1% agarose gel prepared in Tricine buffer for the 1st dimension. Wells were formed at the same time using a homemade comb. The gel was placed in Multiphor II electrophoresis system (GE Healthcare, Velizy-Villacoublay, France) and wells were filled with 2.5 μ L of samples. The samples were then

subjected to the first dimension electrophoresis (600 V, 16 mA, 100 W), freely migrating in the agarose gel band according to their molecular weight. Then, the gel was turned by 90° for the second dimension corresponding to the rocket immunoelectrophoresis (500 V, 12 mA, 100 W), for 3 h 30 min. Gels were dried and stained as described by Walker (2009).

The gels were numerically scanned and area under peaks was integrated using ImageJ software. The first peak on the left side was recognized as native C3, whereas the second peak from the left was attributed to protein fragments derived from C3 cleavage. The % of activation was expressed as the ratio of peak area of cleaved protein regarding the total peak area of C3 native and fragments.

Cytotoxicity test

The cytotoxicity test was performed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) method. Human Umbilical vein/vascular endothelium cells (Huvec, ATCC[®] CRL-1730TM), Mus Musculus Skin Melanoma cells, and Macrophage, Abelson murine leukemia virus-induced tumor cells (Raw 264.7, ATCC[®] TIB-71TM) were maintained cultivated in Dulbecco's modified Eagle's medium (pH 7,4 = 3,7 g/L NaHCO₃) containing 10% of heat-inactivated bovine fetal serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). Human Prostate Carcinoma Cells (PC-3, ATCC[®] CRL-1435TM) were cultivated in RPMI-1640 medium containing 10% of heat-inactivated bovine fetal serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were maintained in a 95% humidified atmosphere of 5% CO₂ at 37°C.

Cells were seeded in 96-well culture plate at 7 10^3 cells/well and incubated overnight to allow attachment. Then, 100 µL of nanoparticles suspension (1.9 10^{-8} - 2 10^{-12} M, three replicates per sample) was added to each well and subsequently incubated for 24 and 48h in

a 95% humidified atmosphere of 5% CO₂ at 37°C. After incubation, 20 μ L of MTT (0.5 mg/ml in PBS) was added for further 2h, the supernatants were removed and 200 μ L of DMSO was added to solubilize the formazan products. Viable cells were quantified by recording the UV absorbance at 570 nm using a plate reader multi-well scanning spectrophotometer (LabSystems Multiskan MS). Cell medium, cell medium with the higher nanoparticles suspension and untreated cells, were performed as controls. The percent of viability was expressed as the relative growth rate (RGR), calculated following the Eq. 1.

$$RGR(\%) = \left(\frac{Abs \ sample - Abs \ control nps}{Abs \ control \ cell - Abs \ control \ medium}\right) x \ 100\%$$
(Eq. 1)

Where the Abs sample is absorbance detected of the cells after treatment with nanoparticles, Abs control nps is the absorbance of the medium with nanoparticles at the same conditions (for eliminate any absorbance of nanoparticles), abs control cell is the cells treated with medium and abs control medium is the absorbance of the medium at the same conditions (to eliminate any absorbance interference).

Cellular uptake of Cy3-labeled PBLG nanoparticles

Cellular uptake of Cy3-labeled PBLG nanoparticles was performed under a modified method described elsewhere (Sulheim *et al.*, 2016). Each cell line were seeded in 24-well culture plates at 100 10^3 cells/well and incubated overnight to allow attachment. Then, 200 µL nanoparticles suspension (2 10^{-9} M, in duplicate) was added to each well and subsequently incubated for 3, 6, 24 and 48h in a 95% humidified atmosphere of 5% CO₂ at 37°C.

After incubation, the medium was removed, the cells were rinsed twice with PBS and 200µL of trypsin was added and incubated for further 5 min. For the Raw 264.7 cell, the

trypsin was substituted by cold EDTA 5%, the cells were detached by gently pipetting till obtain a cloudy suspension. Then 800µL of medium was added to each well and transferred to Eppendorf tubes for a 5 min/200g centrifugation. The supernatant was carefully removed, and the pellet rinsed with 500µL of PBS followed by centrifugation. The pellets were resuspended with 300µL of paraformaldehyde at 2% in PBS pH 7.4. The suspensions were incubated for 15min in cold temperature (fixation). After incubation, the suspensions were rinsed twice as described before. The resultant pellets were resuspended with 300 µL of PBS and stored at 4°C for maximum 5 days. Cells were measured in a BD LSR-fortessa (BD biosciences®). Each nanoparticle system was evaluated three independent times (n= >10,000). The data were analyzed through Flowing Software version 2.5 (Perttu Terho, Turku Centre for Biotechnology, Finland; www.flowingsoftware.com).

In-vitro PBLG nanoparticles size stability

PBLG based nanoparticles were added to 5.5 and 7.4 pH adjusted Milli-Q water at 20 µg nanoparticles/mL approximately and incubated for up to 168h (7 days) at 37°C, respectively. The *in-vitro* degradation was determined following the size and zeta potential measurement by dynamic laser light scattering (Zetasizer 4, Malvern Instruments) at 25°C. The pH adjusted Milli-Q water was not changed during the incubation, after measurement the aliquot was discarded. Each nanoparticle system was evaluated three independent times, and each measurement was made in duplicate.

Hemolysis test

The effect of the PBLG-based nanoparticles on erythrocyte membrane integrity was investigated under the method described elsewhere (Jumaa *et al.*, 2002). Briefly, 100 µL of

erythrocyte stock solution was added into 1 ml of PBLG-based nanoparticles suspension, and incubated for 30 and 60 min at 37°C, respectively. The reaction mixture was ultracentrifuged at 3,500 rpm for 10 min (Thermo Scientific Sorvall ST 16R Centrifuge), and 100µL of the supernatant was dissolved in 2 ml of an ethanol/HCl mixture to dissolve all components and avoid hemoglobin precipitation. An additional ultracentrifugation step was performed. The supernatant absorbance was determined at 398 nm by UV spectrometer (Perkin Elmer Lambda 25 UV/Vis spectrometer). The hemolysis rate (HR) with normal saline as negative control (0% lysis) and distilled water as positive control (100 % lysis) was calculated as follows:

$$HR (\%) = \left(\frac{Abs \ sample - Abs \ negative \ control}{Abs \ positive \ control - Abs \ negative \ control}\right) x \ 100\%$$
(Eq. 2)

Where Abs is the absorbance detected. Each nanoparticle system was evaluated two independent times, and each measurement was made in duplicate. The erythrocyte sample was obtained from C57BL/6 mouse.

Results and discussion

PBLG derivatives

Ring-opening polymerization (ROP) of α -amino acid N-carboxyanhydrides (NCAs) is a well-known synthetic route that has drawn attention due the facility y to prepare a wide diversity of polymers bearing different functionalities (Cheng and Deming, 2012; Wibowo *et al.*, 2014). In the present work, PBLG derivatives were synthesized following different approaches, firstly by ring-opening polymerization (ROP), using a slightly modified method described by Martinez and colleagues (Martínez Barbosa *et al.*, 2007) (Supplementary data1), and then a modification through carbodiimide chemistry (Cauchois *et al.*, 2013; De Miguel *et*

al., 2014) (Supplementary data 2-3).

The polymerizations were followed by FT-IR. The disappearance of absorption bands at ~1855 of C⁵=O, ~1787 cm⁻¹ of C²=O and ~920 cm⁻¹ of C-O-C corresponding to the anhydride ring, respectively, indicated the end of the polymerization reaction (Supplementary data 4) (Pretsch, 2009). An analysis by FT-IR was performed in each polymerization before the addition of the initiator to discard the presence of NCA's auto-polymerization.

After purification and drying process, the PBLG derivates were analyzed by FT-IR, ¹H-NMR and MALDI-TOF. The position of amide absorption bands in the FT-IR spectra are commonly used to characterize the polypeptide conformation. For a polypeptide in an α -helix conformation, the amide I and amide II bands are located at 1656 cm⁻¹ and 1548 cm⁻¹, respectively (Fontaine *et al.*, 2001; Martínez Barbosa *et al.*, 2007; Segura-Sánchez *et al.*, 2010); for a polypeptide in β -conformation, the amide I and amide II bands are located at 1630 cm⁻¹ and 1536 cm⁻¹, respectively. Moreover, an absorption band near to 1260 cm⁻¹ corresponding to the amide III band may be observed in the FTIR spectrum of a polypeptide in a α -helix conformation (Martínez Barbosa *et al.*, 2007). Characteristic peaks were identified in all the polymers corresponding to amide I, II and III bands. Therefore, the exhibited configuration in all polymers corresponds to an α -helix conformation (Supplementary data 5).

The degree of polymerization (DP_n) and subsequently the average molar mass for the polymers contained PEG, could be determined in function of the relative intensities of benzyl groups of the PBLG chain and the ethylene protons of the PEG segment (Jeong *et al.*, 2005; Martínez Barbosa *et al.*, 2007). Due the overlapped peaks in the polymers without PEG segment, the molar mass was determined by MALDI-TOF. The average molar mass determined and the synthesis conditions for each polymer are summarized in the table 1.

Polymer	<u>Dpn_t</u>	NCA (mmol)	[M]0 (mol L ⁻¹)	[I]o (mol L ⁻¹)	Rx time (days)	Yield (%)	Molar mass (g mol ⁻¹)
PBLG ₃₅ -Bz	160	13.8	0.5	0.009	3	82	45 000 ⁽²⁾
PBLG ₃₅ -Cy3		-	-	-	1	85	33 000 ⁽²⁾
PBLG ₃₅ -PEG ₅	160	15.2	0.025	0.009	6	80	41 000 (1)
PEG ₅ -PBLG ₇₀ -PEG ₅	320	15.2	0. 025	0.004	1	80	109 000 ⁽¹⁾
PBLG ₃₅ -PEG ₁₀ -PBLG ₃₅	320	15.2	0.025	0.004	7	90	72 000 ⁽¹⁾
PBLG70-PEG10-PBLG70	640	13.6	0.025	0.002	7	90	114 000 ⁽¹⁾

Table 1. Conditions of BLG-NCA polymerization in DMF at 30 °C

[M]o and [I]o are the initial concentration of NCA and initiator, Dpn_t indicate the theoretical degree of polymerization of each polymerization. Polymers are denominated as follows: Poly(γ -benzyl L-glutamate) referred as PBLG is followed by the name of the initiator (Bz: Benzylamine, PEG: poly(ethylene glycol)), the subscript is indicating the molar mass in KDa. Molar mass determined by (1) RMN, (2) MALDI-TOF.

Preparation and characterization of PBLG-based nanoparticles

Formulation of PBLG-based nanoparticles

The preparation of novel PBLG based nanoparticles was achieved by nanoprecipitation method described elsewhere (Martinez Barbosa *et al.*, 2008; Özcan *et al.*, 2011; De Miguel *et al.*, 2014). Stable and reproducible aqueous suspensions of polymeric nanoparticles were obtained for all systems. Dynamic light scattering (DLS) measurements suggested that the

nanoparticles prepared for all cases have a hydrodynamic diameter under 100 nm and a negative ζ Potential (Table 2).

 Table 2. Physicochemical characterization of PBLG-based nanoparticles by nanoprecipitation

 method in 0.125 % w/v poloxamer solution.

System	Mean hydrodynamic diameter (nm) ^a	PDI	ζ Potential (mV) ^b
PBLG ₃₅ -Bz	63.6 ± 2.26	0.090	-13.4 ± 0.82
PBLG ₃₅ -PEG ₅	42.3 ± 0.74	0.099	-9.8 ± 0.90
PEG ₅ -PBLG ₇₀ -PEG ₅	46.8 ± 0.60	0.085	-11.2 ± 3.14
PBLG35-PEG10-PBLG35	86.4 ± 1.51	0.178	-7.6 ± 1.59
PBLG70-PEG10-PBLG70	64.6 ± 11.43	0.115	-9.8 ± 0.97

^a Measured using dynamic light scattering, n=3. ^b Measured using ZetaSizer 4, Malvern Instruments n=3. Data are the average of 3 batches ± SD, nanoparticles systems in 0.125% w/v of Poloxamer solution. PDI: polydispersion index

A supplementary size analysis was performed by the measure of 100 nanoparticles by the mean of the maximal length and the maximal width of each nanoparticle (Table 3). The sizes founded by TEM are close to those measured by DLS, except for PBLG₃₅-PEG₁₀-PBLG₃₅ where the compact PBLG core could allow a substantial PEG neutral density in the surface.

System	Mean size	Aspect ratio	Equivalent diameter
	(nm)	Г	
PBLG ₃₅ -Bz	46 ± 8	1.2 ± 0.2	41 ± 7
PBLG ₃₅ -PEG ₅	34 ± 3	1.6 ± 0.3	25 ± 3
PEG ₅ -PBLG ₇₀ -PEG ₅	49 ± 4	2.5 ± 0.4	27 ± 4
PBLG35-PEG10-PBLG35	34 ± 4	1.3 ± 0.2	29 ± 4
PBLG70-PEG10-PBLG70	57 ± 6	2.3 ± 0.5	33 ± 6

Table 3. Size determination from TEM measurement as the mean between the maximal length and the maximal width of each nanoparticle, using negative stain (n=100).

Data are the average of 3 batches \pm SD.

The analysis performed by TEM also revealed the aspect ratio and the morphology of the nanoparticles prepared. The TEM micrographs obtained for each system are given in Figure 1.



Figure 1. TEM micrographs at 120 kV obtained from the following PBLG derivatives. (a) PBLG₃₅-Bz, (b) PBLG₃₅-PEG₅, (c) PEG₅-PBLG₇₀-PEG₅, (d) PBLG₃₅-PEG₁₀-PBLG₃₅, (e) PBLG₇₀-PEG₁₀-PBLG₇₀.

The aspect ratio is commonly used to analyze the shape factor on nanoparticles. This parameter quantifies how near the shape of a particle approach the perfect sphere, since the dimension

in a sphere is equal independently of the point selected toward the center. It is calculated by the division of the maximal length and the maximal width measured (Wadell, 1935).

For the PBLG₃₅-Bz and PBLG₃₅-PEG₅ based nanoparticles the morphology appeared as small spheres and rods with an aspect ratio of 1.2 and 1.6, respectively. For the system PEG₅-PBLG₇₀-PEG₅, the morphology appeared as ellipsoidal nanoparticles with an aspect ratio of 2.5. In the case of the systems PBLG₃₅-PEG₁₀-PBLG₃₅ with a morphology roughly spherical and PBLG₇₀-PEG₁₀-PBLG₇₀, with elongated morphology can be explained by the auto-assembled phenomena of the PBLG's core. The small PBLG chain in the system PBLG₃₅-PEG₁₀-PBLG₃₅ can form a compact core leading to a nanoparticle with an aspect ratio of 1.3. In the case of system PBLG₇₀-PEG₁₀-PBLG₇₀, the long PBLG chain is forming an ellipsoidal morphology with an aspect ratio of 2.3.

In the figure 2, the expected nanoparticle morphology is presented, considering PBLG rigid α -helix conformation chain and PEG flexible hydrophilic nature. The resulting nanoparticles could therefore, have a core-shell conformation, presenting a variety of shape, size and surface.



Figure 2. Polymer conformation scheme illustration of PBLG derivatives and theoretical morphology of PBLG-based nanoparticles in presence/absence of poloxamer.

Formulation of Cy3-labeled PBLG-based nanoparticles

According to the purpose of the present work, Cyanine 3 (Cy3)-labeled nanoparticles were prepared with each PBLG derivative presented above. Two set of Cy3-labeled PBLG-based nanoparticles were prepared in presence and absence of poloxamer.

Poloxamer is a tri-block copolymer in conformation ABA, formed by a central hydrophobic chain of polypropylene oxide (PPO) flanked by two hydrophilic chains of poly(ethylene oxide) (PEO) (Santander-Ortega *et al.*, 2006). The PPO central part, can be strongly associated into hydrophobic nanoparticle's surface leading hydrophilic PEO chain Université Paris-Saclay Université Paris Sud/Institut Galien Paris Sud (CNRS UMR 8612), 5 rue Jean-Baptiste Clément, 92290 Châtenay-Malabry, France to form a layer in the surface (Alexandridis and Alan Hatton, 1995; De Sousa Delgado *et al.*, 2001). This effect not only stabilize nanoparticles suspension to avoid aggregation, but also enhance the elastic repulsive forces to reduce nonspecific protein adsorption (De Sousa Delgado *et al.*, 2001). It has been also reported that using poloxamer in the formulation process, can reduce the size of the resulting nanoparticles (Yan *et al.*, 2010). In this work, the nanoparticles that present an exposed hydrophilic surface will adsorpt poloxamer completing a hydrophilic shell. Therefore, it could be evaluated the effect of nanoparticle's shape and surface when interact with cells.

Stable and reproducible aqueous suspensions of Cy3-labeled PBLG-based nanoparticles in the presence of poloxamer were obtained for all systems after optimization. Dynamic light scattering measurements exhibit sizes less than 100 nm for all cases (table 4). In comparison with the PBLG-based nanoparticles, Cy3-labeled PBLG-based nanoparticles present a slightly increment in size even in the presence of poloxamer.

The increment in terms of size by the introduction of Cy3 can be related with a reorganization of PBLG core due Cy3 steric hindrance (Mishra *et al.*, 2000). The covalent bond of Cy3 directly to the PBLG chain maintains the molecule closely at the PBLG core.

In terms of ζ Potential, the Cy3-labeled PBLG-based nanoparticles remained with a negative ζ Potential.

System	Mean hydrodynamic diameter (nm) ^a	PDI	ζ Potential (mV) ^b
PBLG ₃₅ -Bz/ PBLG ₃₅ -Cy3	82 ± 7	0.111	-9 ± 7
PBLG ₃₅ -PEG ₅ / PBLG ₃₅ -Cy3	46 ± 2	0.123	-9 ± 2
PEG ₅ -PBLG ₇₀ -PEG ₅ /PBLG ₃₅ -Cy3	53 ± 2	0.113	-9 ± 1
PBLG35-PEG10-PBLG35/PBLG35-Cy3	99 ± 10	0.192	-4 ± 1
PBLG70-PEG10-PBLG70/PBLG35-Cy3	93 ± 11	0.151	-7 ± 1

Table 4. Physicochemical characterization of Cy3-labeled PBLG-based nanoparticles by nanoprecipitation method in 0.125 % w/v poloxamer solution.

^a Measured using dynamic light scattering, n=3. ^b Measured using ZetaSizer 4, Malvern Instruments n=3. Data are the average of 3 batches ± SD; PDI: polydispersion index.

A supplementary size analysis by TEM was also performed for Cy3-labeled PBLGbased nanoparticles in presence of poloxamer (Table 5). The sizes founded by TEM are close to the sizes measured by DLS, as observed with PBLG-based nanoparticles.

Table 5. Size determination from TEM measurements of Cy3-labeled PBLG-based nanoparticles by nanoprecipitation method in 0.125% poloxamer solution, as the mean of the maximal length of each nanoparticle, using negative stain (n=100). Data are the average of 3 batches \pm SD.

System	Mean size (nm)	Aspect ratio Γ	Equivalent diameter
PBLG ₃₅ -Bz/ PBLG ₃₅ -Cy3	72 ± 16	1.2 ± 0.2	64 ± 13
PBLG ₃₅ -PEG ₅ / PBLG ₃₅ -Cy3	36 ± 5	1.4 ± 0.2	29 ± 5
PEG5-PBLG70-PEG5/PBLG35-Cy3	55 ± 5	1.8 ± 0.3	37 ± 5
PBLG35-PEG10-PBLG35/PBLG35-Cy3	36 ± 5	1.3 ± 0.2	30 ± 5
PBLG70-PEG10-PBLG70/PBLG35-Cy3	61 ± 6	1.9 ± 0.3	40 ± 6

Data are the average of 3 batches \pm SD.

The TEM micrographs obtained for Cy3-labeled PBLG-based nanoparticles are displayed in the Figure 3. Aspect ratio determination was also performed. For the PBLG₃₅-Bz/PBLG₃₅-Cy3 and PBLG₃₅-PEG₅/PBLG₃₅-Cy3 based nanoparticles the morphology appeared as small spheres and rods with an aspect ratio of 1.2 and 1.4, respectively. For the system PEG₅-PBLG₇₀-PEG₅/PBLG₃₅-Cy3, the morphology appeared as ellipsoidal nanoparticles with an aspect ratio of 1.8. PBLG₃₅-PEG₁₀-PBLG₃₅/PBLG₃₅-Cy3 presents



morphology roughly spherical with aspect ratio of 1.3 and PBLG₇₀-PEG₁₀-PBLG₇₀/PBLG₃₅-Cy3 presents an ellipsoidal morphology with aspect ratio of 1.9.



Figure 3. TEM micrographs at 120 kV obtained from Cy3-labeled PBLG-based nanoparticles prepared by nanoprecipitation method in 0.125% w/v poloxamer solution. (a) PBLG₃₅-Bz/PBLG₃₅-Cy3, (b) PBLG35-PEG₅/PBLG₃₅-Cy3, (c) PEG₅-PBLG₇₀-PEG₅/PBLG₃₅-Cy3, (d) PBLG₃₅-PEG₁₀-PBLG₃₅/PBLG₃₅-Cy3, (e) PBLG₇₀-PEG₁₀-PBLG₃₅-Cy3.

The TEM micrographs obtained indicate that the introduction of PBLG₃₅-Cyanine 3 in all systems with the presence of poloxamer does not perturb the general aspect of the final architecture. Therefore, the morphology observed in PBLG-based nanoparticles is preserved even with the introduction of a second polymer.

In the case of Cy3-labeled PBLG-based nanoparticles in absence of poloxamer, the sizes observed were consistently higher (Table 6). Regarding this behavior, PBLG-based nanoparticles in absence of poloxamer were also prepared, as control. As well as observed on Cy3-labeled PBLG-based nanoparticles in absence of poloxamer, slightly higher sizes were founded in the controls (Supplementary data 6).

The presence of poloxamer not only have an effect in terms of size, but it has also a substantial impact on the nanoparticle stability. For the system presenting PBLG₃₅-Bz, it was

not possible to obtain a stable suspension. The aggregation could be as result of the high hydrophobic surface and the increment in size by the presence of PBLG₃₅-Cy3.

Table 6. Physicochemical characterization of Cy3-labeled PBLG-based nanoparticles by

 nanoprecipitation method in Milli-Q water.

System	Mean hydrodynamic diameter (nm) ^a	PDI	ζ Potential (mV) ^b
PBLG35-PEG5/ PBLG35-Cy3	139 ± 1	0.147	-10 ± 0.3
PEG5-PBLG70-PEG5/PBLG35-Cy3	91 ± 1	0.087	-6 ± 0.9
PBLG35-PEG10-PBLG35/PBLG35-Cy3	137 ± 2	0.159	-5 ± 0.9
PBLG70-PEG10-PBLG70/PBLG35-Cy3	126 ± 2	0.074	-12 ± 0.4

^a Measured using dynamic light scattering, n=3. ^b Measured using ZetaSizer 4, Malvern Instruments n=3. Data are the average of 3 batches ± SD; PDI: polydispersion index.

The controls also exhibit a higher negative ζ potential, in comparison with the Cy3nanoparticles, the introduction of Cy3 slightly reduce the negative charge in the surface. Although, for the labeling systems, the ζ potential remains similar.

The TEM micrographs of Cy3-labeled PBLG-based nanoparticles in aqueous suspensions are depicted in the Figure 4. The aspect ratio and morphology analysis were also performed.



Figure 4. TEM micrographs at 120 kV obtained from Cy3-labeled PBLG-based nanoparticles prepared by nanoprecipitation method in Milli-Q water. (b) PBLG₃₅-PEG₅/PBLG₃₅-Cy3, (c) PEG₅-PBLG₇₀-PEG₅/PBLG₃₅-Cy3, (d) PBLG₃₅-PEG₁₀-PBLG₃₅-Cy3, (e) PBLG₇₀-PEG₁₀-PBLG₇₀/PBLG₃₅-Cy3.

For the PBLG₃₅-PEG₅/PBLG₃₅-Cy3 based nanoparticles the morphology appeared as small ellipsoidal nanoparticles with an aspect ratio of 1.7. For the system PEG₅-PBLG₇₀-PEG₅/PBLG₃₅-Cy3, the morphology appeared as ellipsoidal nanoparticles with an aspect ratio of 1.5. PBLG₃₅-PEG₁₀-PBLG₃₅/PBLG₃₅-Cy3 presents morphology roughly spherical with aspect ratio of 1.2 and PBLG₇₀-PEG₁₀-PBLG₃₅-Cy3 presents an ellipsoidal morphology with aspect ratio of 2.1 (Table 7).

Table 7. Size determination from TEM measurement of Cy3-labeled PBLG-based nanoparticles by nanoprecipitation method in Milli-Q water, as the mean of the maximal length of each nanoparticle, using negative stain (n=100). Data are the average of 3 batches \pm SD.

System	Mean size (nm)	Aspect ratio Γ	Equivalent diameter
PBLG ₃₅ -PEG ₅ / PBLG ₃₅ -Cy3	42 ± 6	1.7 ± 0.4	30 ± 0.3
PEG ₅ -PBLG ₇₀ -PEG ₅ /PBLG ₃₅ -Cy3	41 ± 6	1.5 ± 0.3	31 ± 5.4
PBLG ₃₅ -PEG ₁₀ -PBLG ₃₅ /PBLG ₃₅ -Cy3	43 ± 8	1.2 ± 0.2	38 ± 5.6
PBLG70-PEG10-PBLG70/PBLG35-Cy3	67 ± 8	2.1 ± 0.4	42 ± 7.8

Data are the average of 3 batches \pm SD.

Biocompatibility of PBLG based nanoparticles

Complex complement activation

It is well established in the literature that one of the major barriers in the application of nanoparticles is their quick clearance by different pathways of the immunology system. The nanoparticle recognition by the complement system can not only reduce the circulation time and consequently the efficacy of the nanoparticles but also produce a 'pseudo allergic' or inflammatory process in the patient (Moghimi and Szebeni, 2003; Moghimi *et al.*, 2011). It is demonstrated that depending on the nanoparticle's size and the exposed surface, it could have an enhanced recognition and subsequence develop nanotoxicity (Coty *et al.*, 2016).

The activation of the complement system was evaluated by C3 complement protein activation, using 2D immunoelectrophoresis, method described elsewhere (Coty *et al.*, 2016). The results are presented in the figure 5, given as percentages of C3 activation. The systems prepared shows slightly activation activity in comparison with the negative control (solid line-figure 5). For the systems PBLG₃₅PEG₁₀PBLG₃₅ and PEG₅-PBLG₇₀-PEG₅, the activation is slightly higher than the other systems. Comparable results are found in the literature. Hamad and colleagues concluded that the alteration in the nanoparticle surface ("mushroom-like" and brush type) can trigger the activation not only the C3 protein but also different complement system pathways (Hamad *et al.*, 2010). Furthermore, the low C3 protein activation for all systems highlight the ability to be considered as candidates for drug delivery applications.



Figure 5. Determination of the C3 complement protein activation for PBLG based nanoparticles prepared by nanoprecipitation method in 0.125% w/v poloxamer solution. Top: Graph representing the values of C3 percentage activation. The tested surface for all systems corresponds to $2000 \text{ cm}^2/\text{mL}$.

Cellular uptake of nanoparticles

Since de introduction of nanoparticles as nanomedicines, the concept has been evolving to more rational and complex design. This improvement is related with the high demand to overpass the biological barriers in terms of precisely targeting and delivering. As an example of complex design, dendrimers (Li *et al.*, 2010), metal-polymer nanoparticles (Liu *et al.*, 2010; Wen *et al.*, 2013), tri-block polymeric nanoparticles (Tian *et al.*, 2005; Zhang and Zhuo, 2005; Bhattacharjee *et al.*, 2012) and micelle-polymeric nanoparticles (Ko *et al.*, 2009; Zhang *et al.*, 2011; Navarro *et al.*, 2015) are found. Despite the remarkably advance in nanomedicine, rational design is still growing.

In the interest to evaluate the modulated architecture of PBLG-based nanoparticles, from spherical to ellipsoidal shape in presence and absence of PEG in different conformations and length at the surface, two set of Cy3-labeled PBLG-based nanoparticles were selected. Those systems were prepared in presence and absence of poloxamer, a tri-block amphiphilic copolymer capable to be adsorbed on hydrophobic surfaces, creating a protecting hydrophilic layer (Alexandridis and Alan Hatton, 1995; De Sousa Delgado *et al.*, 2001; Santander-Ortega *et al.*, 2006). The effect of poloxamer could isolate at a certain level, the surface impact from the shape characteristic.

HUVEC's were selected as a model of normal vein endothelium, considering that those cells are present during the total nanoparticle's circulation life-time and therefore, they are likely exposed to interact with nanoparticles (Thompson *et al.*, 2013). Immune system is a highly complex system, composed by several pathways. It is well documented that the recognition by the immune system lead to a quick nanoparticle's clearance, limiting the

circulation life-time and therefore the nanoparticle's efficiency as drug delivery system Université Paris-Saclay Université Paris Sud/ Institut Galien Paris Sud (CNRS UMR 8612), 5 rue Jean-Baptiste Clément, 92290 Châtenay-Malabry, France (Sharma *et al.*, 2010; Firdessa *et al.*, 2014). Macrophage (Raw 264.7) were selected due their excellent capacity to recognize and eliminate foreign particles. B16 and PC-3, were selected as models of circulating tumor cells due their remarkably capacity to develop metastasis process (Danila *et al.*, 2007; Clawson *et al.*, 2012).

The cellular uptake of PBLG-based nanoparticles in presence of poloxamer is depicted in the figure 6. Through the analysis, it can be observed a general trend in cellular uptake ratio, orderly in first place by the aspect ratio and secondly by the PEG conformation in the surface. The tendency is preserved through each cell line evaluated.



Figure 6. I) Cellular uptake of PBLG-based nanoparticles at 2nM in Endothelial cells (HUVEC, \blacksquare), Prostatic cancer cells (PC-3, \bullet), Melanoma cancer cells (B16, \blacktriangle) and Macrophage-type cells (Raw 264.7, \diamond) at 24h and 48h after exposition, expressed in terms of nanoparticle's aspect ratio (Γ) over mean fold fluorescence increase over control. The nanoparticles evaluated are PBLG₃₅-Bz (Γ =1.1), PBLG₃₅-PEG₁₀-PBLG₃₅ (Γ =1.3), PBLG₃₅-PEG₅ (Γ =1.), PEG₅-PBLG₇₀-PEG₅ (Γ =1.8), and PBLG₇₀-PEG₁₀-PBLG₇₀ (Γ =1.9), each nanoparticle is labeled with ~1% w/w of PBLG₃₅-Cy3, respectively (n=3). II) confocal images of merge transmission channel and red channel of PBLG₃₅-Bz/PBLG₃₅-Cy3 in A-1) PC-3, A-2) B16 and A-3) Raw 264.7 cell lines and PBLG₃₅-PEG₁₀-PBLG₃₅/PBLG₃₅-Cy3, in B-1) PC-3, B-2) B16 and B-3) Raw 264.7, after 48h in all cases. (Cy3 = Cyanine 3). Note: For most

experimental points, the standard deviation was very small and could be not visually evident on the graphic.

In a general frame, it was observed a substantial difference within cell lines. It is positively found that endothelial cells are poorly interacting with PBLG-based nanoparticles despite their architecture. For example, PBLG₃₅-Bz system that is highly associated with macrophage-type cell at 48h showing a 127-fold increment over control, only present a 3.7fold increment in HUVEC cells at the same conditions. It is also found that the interaction nanoparticles/cells could be modulated by PEG conformation. The presence of hairy-type PEG conformation decreases the nanoparticle/cell interaction. For example, PEG₅-PBLG₇₀-PEG₅ system with an aspect ratio of 1.8, bearing PEG chains in "hairy-type" conformation in the extremities present 0.6-fold. In contrast, their counterpart, PBLG₇₀-PEG₁₀-PBLG₇₀ system with an aspect ratio of 1.9, bearing "loop-type" PEG conformation in the extremities present 1.5fold over control at 48h. Similar results were found in the literature for those systems bearing PEG in the surface. Ehrenberg and colleagues (Ehrenberg et al., 2009) evaluated the influence of protein/nanoparticle association with HUVEC. The authors compared eight different surface functional groups. Among their results, they established that the nanoparticles bearing PEG in the surface associated least in comparation to those bearing $COOH_{100}$, amine, lysine, etc. They determined that nanoparticles bearing charged functional groups in the surface could associated more to HUVEC cells, due to electrostatic interactions.

In other hand, Davda and Labhasetwar (2002), evaluated the cellular uptake by endothelial cells of poly(DL-lactide-co-glycolide) (PLGA) nanoparticles. The authors determined that PLGA nanoparticles are quickly uptake by HUVEC and their cellular uptake is depending on nanoparticle's concentration and incubation time. Even in the lowest nanoparticle concentration, PLGA nanoparticles were efficiently captured by HUVEC. The PLGA nanoparticles used by Davda and colleagues, could be related with PBLG₃₅-Bz system characteristics. However, comparing PLGA nanoparticles and PBLG₃₅-Bz system, our system present low interaction/capture by HUVEC even at 48h of incubation. Considering the results obtained, the low interaction/association of PBLG-based nanoparticles with HUVEC cells, positively indicated that PBLG-based nanoparticles could avoid unspecific accumulation in endothelial cells, remaining available for their selected target, enhancing their circulation life-time and reduce side-effects.

Regarding cancer cell lines, melanoma cell line (B16) exhibit greater cellular uptake than prostate cells (PC-3) of PBLG-based nanoparticles prepared in presence of poloxamer. In this work, PBLG₃₅-Bz system is used as positive reference. This system presents 17.6-fold increment in B16 compared with 13.2-fold in PC-3 at 48h. In comparison with nanoparticles sharing similar morphology and aspect ratio, PBLG₃₅-PEG₁₀-PBLG₃₅ system, presents 7.2-fold in B16 and 1.6-fold in PC-3 at the same conditions. Following PEG "mushroom-like" conformation, PBLG₇₀-PEG₁₀-PBLG₇₀ system presents 5.4-fold in B16 compared with 1.7fold in PC-3, similar to his homologous. For those with "hairy" PEG conformation, PEG₅-PBLG₇₀-PEG₅ system presents 8-fold higher uptake in B16 than PC-3, followed by PBLG₃₅-PEG₅ system presenting a 13-fold higher in P16 compared with PC-3 at 48h.

Thus, based in our results on PBLG-based nanoparticles prepared in presence of poloxamer, the nanoparticle's shape and furthermore the aspect ratio is a key parameter for cellular uptake regardless the cell lined evaluated. Several authors highly the importance of this parameter in nanoparticles design. It has been correlated low cellular internationalization in high aspect ratios due to the difficulty for the cell to wrap the nanoparticle, especially when

the orientation of the major axis is facing the cell membrane (Champion *et al.*, 2007; Verma and Stellacci, 2010; Venkataraman *et al.*, 2011; Toy *et al.*, 2013; Deng and Changyou, 2016). In addition, cellular uptake can be regulated at certain level by the conformation of PEG chains at nanoparticle's surface. In summary, PBLG-based nanoparticles prepared in presence of poloxamer exhibit greater affinity for Raw 264.7>B16 cells>PC-3 >HUVEC cells. It is important to remark that the systems bearing "hairy-type" PEG present the lowest interaction levels. However, this behavior could be easily modified through the coupling of functional groups to the PEG chain.

In the section II of figure 6, confocal images of PBLG₃₅-Bz and PBLG₃₅-PEG₁₀-PBLG₃₅ systems, meaning the systems with highest cellular uptake level after 48h in PC-3, B16 and Raw 264.7 cell lines are depicted. In each case, positive fluorescence level is found. Spread accumulation as small dots with different sizes is observed. None of those dots were found in the well-defined nucleus, these results are in concordance with the literature. It has been reported that only nanoparticles smaller than 40nm could enter to the nucleus (Deng and Changyou, 2016). Considering the aspect of the accumulation and the nanoparticles sizes, an uptake mediated by endocytosis may be the pathway used by the evaluated cell lines. Several authors reported a size-limited cellular uptake for nanoparticles. Macropinocytosis (large nanoparticles), clathrin mediated endocytosis (~100nm vesicle size) and caveolae mediated endocytosis (~60-80nm vesicle size) are some pathways suggested, regarding if the nanoparticle could fit into the vesicle (Bhattacharjee *et al.*, 2012; Kou *et al.*, 2013; Deng and Changyou, 2016). Further studies should need be performed to determine the explicit endocytosis pathway by PBLG-based nanoparticles. Following with the analysis, the second set of PBLG-based nanoparticles prepared in absence of poloxamer were evaluated at the same conditions. It is important to recall that an increment in size of nanoparticles was observed (i.e. ~90 to 140nm). Furthermore, PBLG₃₅-Bz system was unstable due the high hydrophobicity surface and the presence of Cy3, both factors causing nanoparticle aggregation. Overall, it is observed in the remaining systems, that general aspect ratio (table 6) and morphology (figure 4) is preserved similar in comparison to those prepared in presence of poloxamer.

The cellular uptake results of the second set are depicted in the figure 7. It is observed immediately a shift in the cellular uptake ratio beginning at 24h and clearly increased at 48h. Systems with an aspect ratio of 1.2, 1.5 and 1.7 preserved similar uptake ratios compared with their homologous in presence of poloxamer. Moreover, the system with an aspect ratio of 2.1 presents a substantial increment through each cell line evaluated. It is evident the highly complex cellular uptake process.

Meanwhile the first set is governed mainly by the aspect ratio, the second set is the conjunction of aspect ratio and surface's characteristic that implies an enhanced effect. According with the literature, this tendency could be the result of poloxamer protective layer elimination, that is formed as a "cloud" at nanoparticle's surface (Alexandridis and Alan Hatton, 1995; Santander-Ortega *et al.*, 2006; Yan *et al.*, 2010).

Furthermore, substantial differences within cell lines and nanoparticle's architecture was observed. As inferred in the first set, it is positively found low uptake by endothelial cells at 24 and 48h after exposition. Therefore, non-substantial poloxamer effect was determined. These results reaffirm the evidence that PBLG-based nanoparticles would remain available to interact with the selected target once administrated.


Figure 7. I) Cellular uptake of PBLG-based nanoparticles at 2nM in Endothelial cells (HUVEC, \blacksquare), Prostatic cancer cells (PC-3, \bullet), Melanoma cancer cells (B16, \blacktriangle) and Macrophage-type cells (Raw 264.7, \diamond) at 24h and 48h after exposition, expressed in terms of nanoparticle's aspect ratio (Γ) over mean fold fluorescence increase over control. The nanoparticles evaluated are PBLG₃₅-PEG₁₀-PBLG₃₅ (Γ =1.2), PEG₁₀-PBLG₇₀-PEG₁₀ (Γ =1.5), PBLG₃₅-PEG₅ (Γ =1.7) and PBLG₇₀-PEG₁₀-PBLG₇₀ (Γ =2.1), each nanoparticle is labeled with ~1% w/w of PBLG₃₅-Cy3, respectively (n=3). II) confocal images of merge transmission channel and red channel of PBLG₃₅-PEG₁₀-PBLG₃₅/PBLG₃₅-Cy3 system in A-1) PC-3, A-2) B16 and A-3) Raw 264.7 and PBLG₇₀-PEG₁₀-PBLG₇₀/PBLG₃₅-Cy3 system in B-1) PC-3, B-2) B16 and B-3) Raw 264.7, after 48h in all cases. (Cy3 = Cyanine 3). Note: For most experimental points, the standard deviation was very small and could be not visually evident on the graphic.

The macrophage-type cell initially recognized PBLG₇₀-PEG₁₀-PBLG₇₀ system even with an aspect ratio of 2.1 above PBLG₃₅-PEG₁₀-PBLG₃₅ system with an aspect ratio of 1.2. However, it is PBLG₃₅-PEG₁₀-PBLG₃₅ system that presents the highest rate at 48h, with an uptake of 117-fold over control in comparison to 73.8-fold of PBLG₇₀-PEG₁₀-PBLG₇₀ system. Although, both systems present a "mushroom-like" PEG conformation, the final architecture of each system is highly different. In PBLG₃₅-PEG₁₀-PBLG₃₅ system, the short hydrophobic PBLG chains are oriented to form a compact center leading a high dense PEG layer at the surface. In contrast, PBLG₇₀-PEG₁₀-PBLG₇₀ system, the long hydrophobic PBLG chains are stacked toward the center forming an ellipsoidal center instead of a thin elongated center, and therefore PEG chains are oriented at the extremities of the major axis. The ellipsoidal center is likely exposed to interact with the cell membrane but then the difficulty to wrap the nanoparticle is limiting their engulfment. Difficulty that is not presented in the spherical PBLG₃₅-PEG₁₀-PBLG₃₅ system.

This phenomenon is also observed in B16 and PC-3. In contrast to the first set, PC-3 is presented the highest uptake ratio in comparison to B16. Even though, PBLG₃₅-PEG₁₀-PBLG₃₅ system is preserving similar uptake rates to the one of the first set, PBLG₇₀-PEG₁₀-PBLG₇₀ and PBLG₃₅-PEG₅ system are consistently higher. PBLG₇₀-PEG₁₀-PBLG₇₀ system in absence of poloxamer is ~64-fold higher in PC-3 and ~14-fold higher in B16 in comparison to the one prepared in presence of poloxamer at 48h. The same tendency is observed in PBLG₃₅-PEG₅ system. In absence of poloxamer, PBLG₃₅-PEG₅ system prepared in presence of poloxamer. PEG₅-PBLG₇₀-PEG₅ system remains similar to the uptake rates in presence of poloxamer. The poorly interaction of PEG₅-PBLG₇₀-PEG₅ system could be a combination of the highly flexible PEG chains at the major axis extremities and the aspect ratio. Despite this low cellular uptake, PEG₅-PBLG₇₀-PEG₅ system prepared in absence of poloxamer exhibit a 10-fold increment in PC-3 at 48h.

As inferred in the first set, PBLG-based nanoparticles prepared in presence of poloxamer exhibit greater affinity for Raw 264.7>B16 cells>PC-3 >HUVEC cells. However, in the second set, PBLG nanoparticles prepared in absence of poloxamer, the trend is towards PC-3>B16>Raw 264.7>HUVECs. Although, PBLG₃₅-PEG₁₀-PBLG₃₅ system is presenting the same trend as if it is prepared in presence of poloxamer. This evidence reaffirms the

theoretical distribution of PEG chains in the surface. Since PBLG block with a molar mass of 35 000 gmol⁻¹, representing ~25 nm in size (value calculated from the total molar mass of PBLG block and the length of the amino acid unit (Klok *et al.*, 2000)) could self-aggregate into a compact core, the PEG chains could remain dispersed in the surface. Thus, considering the flexibility of PEG chain, few poloxamer chains could be attached at the surface. Therefore, PBLG₃₅-PEG₁₀-PBLG₃₅ system could remain similar in presence or absence of poloxamer. Considering the trends followed by each set, it can be inferred that PBLG-based nanoparticles prepared in absence of poloxamer could modulate more appropriated their fate in the body.

As performed in the first set of PBLG-based nanoparticles, confocal images of PBLG₃₅-PEG₁₀-PBLG₃₅ and PBLG₇₀-PEG₁₀-PBLG₇₀ systems, corresponding to highest uptake rates in PC-3, B16 and Raw 264.7 cell lines are depicted in the section II of figure 7. Positive fluorescence is observed in each cell line. Similar spread dots accumulation with a range of sizes is observed as those of the first set. The apparently higher fluorescence level in Raw 264.7 treated with PBLG₇₀-PEG₁₀-PBLG₇₀ system compared with PBLG₃₅-PEG₁₀-PBLG₃₅ system could be explained by the core size of each system. Since the core of PBLG₃₅-PEG₁₀-PBLG₃₅ system is smaller (44nm) compared to PBLG₇₀-PEG₁₀-PBLG₇₀ system (67nm), less molecules of Cy3 could be present, due to less PBLG₃₅-Cy3 chains assembled in the core.

Considering the results obtained in the second set, PBLG-based nanoparticles presenting a surface/shape variety, the cellular uptake also followed a tendency ordered by the aspect ratio, but it could be modulated through a hydrophobic/hydrophilic balance.

Cytotoxicity test

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is an extended method used to determine the *in-vitro* cytotoxicity or chemosensitivity of a wide range of substances in cells (Burton, 2005; Van Meerloo *et al.*, 2011; Tolosa *et al.*, 2015). MTT assay is based on the conversion of MTT into formazan crystals by mitochondrial activity in metabolically active cells (Van Meerloo *et al.*, 2011). According to the literature, the appropriate selection of the cells, the sample preparation and the use of the right dose are crucial considerations for assessment the cytotoxicity level of nanoparticles by means of *in-vitro* assays (Kong *et al.*, 2011).

The concentrations tested in MTT assay were calculated based in the number of nanoparticles in each suspension. To determine the molarity, the nanoparticle volume, PBLG density and Avogadro number were considered for the calculation. The objective of calculating the concentration in molarity terms is to homogenizer the number of nanoparticles per concentration, meaning that even though the system PBLG₃₅-PEG₅ with a size by DLS of ~42 nm will be equal in number to a system with bigger size as the case of PBLG₃₅-PEG₁₀-PBLG₃₅ with a size by DLS of ~87 nm.

The results obtained after MTT determination exhibit that HUVEC cells remains with a favorable cell viability beyond 80% at 24h and 48h after exposition (Supplementary data7). This evidence is reaffirmed by the results obtained through cellular uptake analysis, in which we observe low cell interactions with nanoparticles regardless the general nanoparticle's architecture. Hence, PBLG-based nanoparticles would avoid non-specific accumulation in epithelium cells and therefore, they will persist in circulation.



In terms of immune recognition, the nanoparticles were tested in RAW 264.7 cells, a macrophage-type cell. The MTT evaluation exhibit that the systems containing PEG and poloxamer F68 in the surface, present a cellular viability above 80%. For the system PBLG₃₅-Bz despite the presence of poloxamer is highly recognized by Raw 264.7 with an IC₅₀ of 14nM (Supplementary data 8). In comparison with PBLG₃₅-PEG₁₀-PBLG₃₅ system, the high density of PEG may avoid the recognition even if both systems have an aspect ratio below 1.4.

The *in vitro* cytotoxic activity of PBLG-based nanoparticles in melanoma is depicted in the figure 8. The behavior of systems with a spherical morphology, meaning to an aspect ratio of ~1.1 to 1.3, presents a higher interaction with cells, as observed in the cellular uptake analysis. PBLG₃₅-Bz and PBLG₃₅-PEG₁₀-PBLG₃₅ systems presents an IC₅₀ of ~11nM and ~4nM, respectively. For those systems with an aspect ratio more than 1.5, a cell viability remains above 80% at 24h and a slightly reduction at 48h. The cell recovery observed at 48h in comparison to 24h related with the fast growth of melanoma cancer cells.



Figure 8. MTT cytotoxic assay of PBLG-based nanoparticles on B16 GFP+ cell line after a) 24 and b) 48h after exposition with PBLG-based nanoparticles prepared by nanoprecipitation method in 0.125% w/v poloxamer F68 solution. The concentration of PBLG-based nanoparticles is calculated in number of particles as molarity, using Avogadro number. (n = 3)

In contrast, prostate cancer cells evidence a higher cytotoxicity after nanoparticle exposition. As inferred in the figure 9, the system PBLG₃₅-Bz present an IC₅₀ of 2nM. This high cytotoxicity level is also confirmed by cellular uptake analysis. The spherical morphology and the size of this system enhance cell interaction as explained by Toy and colleagues (Toy *et al.*, 2013). Following this concept, PBLG₃₅-PEG₁₀-PBLG₃₅ system, with an aspect ratio of 1.3, exhibit also a high cell interaction as observed by flux cytometry, but in terms of *in vitro* cytotoxicity, its lower than PBLG₃₅-Bz system (IC₅₀ of 6nM). This behavior could be related with the presence of "mushroom-like" PEG conformation at the surface. As it is also observed in the system PBLG₇₀-PEG₁₀-PBLG₃₅ with an IC₅₀ of 5nM. In comparison, PBLG₃₅-PEG₅ and PEG₅-PBLG₇₀-PEG₅, both with hairy type PEG conformation, exhibit an IC₅₀ of 4nM.



Figure 9. MTT cytotoxic assay of PBLG-based nanoparticles on PC-3 cell line after a) 24 and b) 48h of exposition with PBLG-based nanoparticles prepared by nanoprecipitation method in 0.125% w/v poloxamer F68 solution. The concentration of PBLG-based nanoparticles is calculated in number of particles as molarity, using Avogadro number. (n = 3).

As performed above, it was also evaluated PBLG-based nanoparticles prepared in absence of poloxamer. Due the spontaneous aggregation behavior, it was only possible to achieve a concentration of 2nM for each preparation. The MTT determination at that concentration evidenced a cell viability above 80% at 24h and 48h after exposition despite the system architecture or cell type (Data not show).

Hemolysis test

Biocompatibility is one of the aims in terms of DDS design. In intravenous applications, the red blood cells are the first element to be exposed to DDS. Considering the highly sensitivity of erythrocytes membrane, several factors can trigger the hemolysis (Manaargadoo-Catin *et al.*, 2016). Depending of the hemolysis level, adverse physiological conditions can

be developed (Rother *et al.*, 2005). Therefore, the evaluation of hemolysis activity of DDS gives valuable information of biocompatibility and toxicity aspects.

The hemolysis activity (HA) of PBLG-based nanoparticles was studied by the measurement of hemoglobin release at 30 and 60 min of incubation after exposition with nanoparticles diluted in PBS at pH 7.4. The set of concentrations were fixed from those used in MTT assays (19 - 3nM) to 0.2nM. In the figure 10, the results obtained are presented.

The first determination at 30min exposed that nanoparticles with an aspect ratio higher than 1.3, consistently exhibit a hemolysis activity (HA) under 5%, even at 19nM. Thus, nanoparticles systems based on PBLG₃₅-PEG₅, PEG₅-PBLG₇₀-PEG₅ and PBLG₇₀-PEG₁₀-PBLG₇₀, present a HA within the requirements of the hemolytic test for biomaterials (Rao and Sharma, 1997), and can be compared with the biocompatibility determined by hemolysis activity of divers DDS among the literature (Morey *et al.*, 2004; Li *et al.*, 2009; Múzquiz-Ramos *et al.*, 2013). In contrast, those with an aspect ratio less than 1.3, exhibit a higher activity. Particularly in the system based on PBLG₃₅-PEG₁₀-PBLG₃₅ with an HA₅₀ of 11.8nM.

The tendency observed at 30 min is preserved at 60 min. Although, higher HA level was observed for each system. This behavior can be related with longer exposition time with a fixed quantity of erythrocytes. The same concentration-exposition time dependency is reported by Jing Zhang and colleagues in spherical oleic acid-grafted chitosan nanoparticles (Zhang *et al.*, 2012). Even though the percentage observed overpass the recommended standard of 5% among concentrations further than 2nM, it is necessary to considerer that once the nanoparticles are injected via intravenous pathway, a dilution process will start spontaneously. Therefore, it can be expected a lower or similar effect as observed at 30 min.



Figure 10. Hemolysis activity of PBLG-based nanoparticles. a) 30 min and b) 60min after exposition.

Furthermore, considering the extended HA level obtained at 60 min for each case, it was possible to calculate the corresponding HA₅₀, meaning the concentration to obtain 50% of hemolysis. For the system based in PBLG₃₅-Bz and PBLG₃₅-PEG₅, a HA₅₀ of 11nM and 10nM was found. In the cases of PEG₅-PBLG₇₀-PEG₅ and PBLG₃₅-PEG₁₀-PBLG₃₅ systems, a HA₅₀ of 7nM and 9nM was determined, those exhibit the highest activity. For the system based on PBLG₇₀-PEG₁₀-PBLG₇₀, a HA₅₀ of 14nM was found, corresponding with the lowest HA level within the systems evaluated.

In-vitro PBLG nanoparticles stability

The evaluation of particle degradation is a crucial step for asset the system stability. If the system is degraded during their circulation time, the nanoparticle will not be available to interact with the target site and consequently, it could deliver the encapsulated drug at Université Paris-Saclay Université Paris Sud/Institut Galien Paris Sud (CNRS LIMR 8612). 5 rue Jean-Bantiste Clément, 92290

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unspecified places developing the undesired side effects. In the present work, it was evaluated the nanoparticle degradation by pH effect. Two pH were selected, normal human blood pH 7.4 (Severinghaus, 1958) and cancer microenvironment pH 5.5, that is slightly more acid than normal tissue (Gerweck e Seetharaman, 1996). The degradation assay was achieved by the measurement of nanoparticle size and ζ Potential by DLS technique, described above.

The sizes measured for PBLG based nanoparticles at pH 7.4 are consistently conserved for up of 168 h (Supplementary data 9). In the case of pH 5.5, all PBLG based nanoparticles except for PBLG₃₅-Bz system, conserved consistently the size during the period evaluated (SD10). The increased size observed at prolonged times could be the result of aggregation due to the size and hydrophobic surface of PBLG. As it was described above, this behavior can be modified by the presence of PEG chains as a protected shell. The results are a favorable indication of the system stability at physiological pH and tumor microenvironment.

Conclusions

Nanoparticles as nanomedicines are transitioning to more rational and complex design to overpass the biological barriers in terms of precisely targeting and delivering. PBLG derivatives that were successfully synthesized by ring-opening polymerization method are following this transitional line. They highlight their capacity to provide a wide variety of copolymers just by changing the molecular initiator and controlling the synthesis conditions. The resultant nanoparticle systems, display a variety of size, surface and shape characteristics. Therefore, it can be inferred that the distribution of the hydrophobic and hydrophilic chain parts in the polymer structure and the functional groups presented, could modulate the architecture obtained. This allowed us to design new polymeric derivatives guiding to a desired architecture.



Low cytotoxicity, hemolysis activity and complement activation levels, highlight their good biocompatible profile. Through our results, it was determined that a concentration of 2nM, equivalent to 1.44 10²¹ nanoparticles/mL, could be an optimal concentration for future nanomedicine applications.

Two set of Cy3-labeled PBLG-based nanoparticles presenting a size, shape and surface variety were developed to evaluate their cell/nanoparticle interaction. The resultant nanoparticles conserved the general architecture observed in those without fluorescent-dye polymer. This evidence highlights the capacity to produce nanoparticles composed by two or more polymers wearing different featuring.

Based in our results, the tendency in cellular uptake of Cy3-labeled PBLG-based nanoparticles with similar surface is ordered by the aspect ratio. Meanwhile, in nanoparticles presenting a surface/shape variety, the cellular uptake also followed a tendency ordered by the aspect ratio, but it could be modulated through a hydrophobic/hydrophilic balance of the proposed surface. Furthermore, low endothelial uptake ratio is found as a positive indication of low non-specific interaction toward normal tissue, allowing PBLG-based nanoparticles not merely to reduce secondary effects but also to have longer circulation life-time once administrated. In addition, PBLG₃₅-PEG₅ and PEG₅-PBLG₇₀-PEG₅ systems presented the best profiles among the systems evaluated in this work.

In summary, PBLG-based nanoparticles present attractive characteristics to nanomedicine applications. Their potential use is not merely based on their well-defined biocompatible profile or their stability through time, it is their potential capacity to reorganize



and modulate their architecture and fate in the body that make them solid candidates for rational-designed nanomedicines.

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Supplementary data

RATIONAL DESIGNED POLY(γ-BENZYL L-GLUTAMATE) NANOPARTICLES FOR NANOMEDICINE APPLICATIONS IN CANCER TREATMENT

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BLG-NCA

SD1. Polymerization of γ -benzyl-L-glutamate N-carboxyanhydride leading to PBLG di- and tri-block copolymers.



SD2. Chemical pathway for the synthesis of PEG₅-PBLG₇₀-PEG₅.



SD3. Chemical pathway for the synthesis of PBLG₃₅-Cy3.

NH-

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-H₂N-PEG-NH₂

Hab



SD4. FTIR spectrum of PBLG₃₅-Bz polymerization film. (I) T0min, (II) T60min.



SD5. FTIR spectrum of PBLG copolymers powders. PBLG₃₅-Bz (V), PBLG₃₅-PEG₅ (IV), PBLG₇₀-PEG₁₀PBLG₇₀ (III), PBLG₃₅-PEG₁₀PBLG₃₅ (II) and PEG₅-PBLG₇₀-PEG₅ (I).

System	Mean hydrodynamic diameter (nm) ^a	PDI	ζ Potential (mV) ^b
PBLG ₃₅ -PEG ₅	57 ± 10	0.135	-13.1 ± 1.1
PEG ₅ -PBLG ₇₀ -PEG ₅	98 ± 19	0.129	-18.7 ± 0.9
PBLG35-PEG10-PBLG35	82 ± 2	0.100	-9.2 ± 3.8
PBLG70-PEG10-PBLG70	73 ± 1	0.092	-16.5 ± 0.5

^a Measured using dynamic light scattering, n=3. ^b Measured using ZetaSizer 4, Malvern Instruments n=3. Data are the average of 3 batches ± SD, nanoparticles systems in aqueous suspension. PDI: polydispersion index

SD6. Physicochemical characterization of PBLG-based nanoparticles by nanoprecipitation method in aqueous suspension.



SD7. MTT cytotoxic assay of PBLG-based nanoparticles on Huvec cell line after a) 24 and b) 48h of exposition with PBLG-based nanoparticles prepared by nanoprecipitation method in 0.125% w/v poloxamer F68 solution. The concentration of PBLG-based nanoparticles is calculated in number of particles as molarity, using Avogadro number. (n = 3)

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SD8. MTT cytotoxic assay of PBLG-based nanoparticles on Raw 264.7 cell line after a) 24 and b) 48h of exposition with PBLG-based nanoparticles prepared by nanoprecipitation method in 0.125% w/v poloxamer F68 solution. The concentration of PBLG-based nanoparticles is calculated in number of particles as molarity, using Avogadro number. (n = 3)



SD9. In vitro degradation of PBLG based nanoparticles at pH= 7.4, 37°C. Measures were performed with a Zetasizer 4, Malvern Instruments. Experiments and measurements were made in triplicate.





SD10. In vitro degradation of PBLG based nanoparticles at pH= 5.5, 37°C. Measures were performed with a Zetasizer 4, Malvern Instruments. Experiments and measurements were made in triplicate.

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CHAPITRE IV

RATIONAL DESIGN OF IMMUNONANOPARTICLES CONCEIVED FOR INTERCEPTING MELANOMA CTCS WITHIN THE BLOOD STREAM

RATIONAL DESIGN OF IMMUNONANOPARTICLES CONCEIVED FOR INTERCEPTING MELANOMA CTCS WITHIN THE BLOOD STREAM

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Abstract

The aims of this work were to develop and characterize nanoparticles and immunonanoparticles conjugated with MART-1 antibody, as well as to evaluated their *in vitro* behavior by complement activation, cytotoxicity and cellular uptake assays. Polymeric nanoparticles were developed through the nanoprecipitation method of polymers derived from poly(γ -benzyl-L-glutamate) (PBLG) and the immunonanoparticles conjugated with MART-1 antibody, specific for melanoma cells, were obtained through the streptavidin-biotin binding. The conjugation of this antibody on the nanoparticles surface was evaluated by *western blot*. The nanoparticles were characterized and evaluated *in vitro* in B16-GFP melanoma cells and human umbilical vein endothelial cells (HUVECs) and the complement activation was investigated by bidimensional immunoelectrophoresis.

The nanoparticles presented sizes between 20 and 100 nm and negative surface charge (-3 to -30 mV). The conjugation of antibody on the nanoparticle surfaces was detected by the *western blot* technique and confirmed by the changes in particle size and surface charge. The developed nanoparticles were not able to activate the complement system being considered long blood circulation. Regarding the *in vitro* analysis, the particles presented a low cytotoxicity when tested in B16-GFP and HUVECs cells. In the cell capture assays, the immunonanoparticles, containing a specific antibody for the recognition of the overexpressed antigen in melanoma cells, showed an increase of 40 to 50% in the uptake for these cells, indicating a specificity of this nanocarrier.

These results suggested a promising application of PBLG-based nanoparticles coupled with MART-1 antibody to be used as systemic drug-delivery systems for melanoma targeting approach and shed light to the optimization of important parameters on the formulation of PBLG immunonanoparticles.

Keywords: Polymeric nanoparticles, PBLG, MART-1, immunonanoparticles, melanoma.

Melanoma, one of the deadly skin cancer, is characterized specially by their high multidrug resistance, high metastatic potential and low survival rates (Slominski et Carlson, 2015). The melanoma incidence increased rapidly over the last 50 years and the 5-year survival rate for patients with advanced cutaneous melanoma was 9 to 28% in Europe (Markovic et al. 2007; Svedman et al., 2016). The high potential of a malignant melanoma to develop a metastatic disease is due to the natural heterogeneity of melanoma cells, leading to a lack of effective chemotherapy administered after surgery (Gray et al., 2015).

Conventional chemotherapy is largely ineffective for the treatment of metastatic melanoma cancer and generally have several limitations as the high toxicity to healthy cells, low target specificity and poor pharmacokinetic properties of the chemotherapeutic agents (Xie et al., 2015). Over the past two decades, the use of nanotechnology has been considered as a promising approach to surpass these drawbacks related to conventional chemotherapy and increase the clinical anticancer efficacy (Chowdhury et al., 2016; Silva et al., 2016). Among the nanotechnology-based drug delivery systems, the polymeric nanoparticles offers several advantages for oncology applications, mainly due to their tailoring properties (Kamaly et al., 2016). The chemical surface modification of the polymeric nanoparticles can provide benefits for anticancer therapy, increasing the local concentrations of chemotherapeutic agents, minimizing dose and side effects (Mora-Huertas et al., 2010; Parhi et al., 2012).

Different polymers can be used for nanoparticle formation, but the peptide-based polymers have gained attention in cancer research due to their suitable properties for biomedical applications. These features can include polymer biodegradability, the ability to carrier high drug loading, chemical stability and ability to control their physicochemical and biological characteristics (Duro-Castano et al., 2014). Peptide-based polymers, including poly(γ -benzyl-L-glutamate) (PBLG) has emerged as a promising choice to prepare polymeric nanoparticles with tailorable structures (Cauchois et al., 2013). The PBLG can adopt rigid α -helices structures and have the ability to form self-assembled nanoparticles by the simple nanoprecipitation method, with different sizes and shapes, depending on the polymer molar mass (de Miguel et al., 2014; Ponchel et Cauchois, 2016). Besides these attractive chemical properties, the PBLG copolymers can be tailored to allow the attachment of target moieties on the surface of nanoparticles for active drug targeting to cell membranes or intracellular levels. This strategy aim to increase the receptor mediated-endocytosis of nanoparticles by cancer cells and/or direct target to cytoplasmic organelles for gene therapy (Chou et al., 2011; Pan et al., 2012; Zou et al., 2016). Among different classes of molecules used for this purpose, the surface functionalization of nanoparticles with antibody target moieties for specific recognition of biomarkers overexpressed in cancer cells is one of the most well-described strategy (Manjappa et al., 2011; Lammers et al., 2012; Liu et al., 2014).

In the case of melanoma biomarkers, MART-1 is the antigen recognized by T-cells, which is encoded by melanocyte-specific genes involved with the differentiation and production of melanin. MART-1 is overexpressed in the plasma membrane and in the endoplasmic reticulum of melanoma cancer (Chen et al., 1996; Riker et al., 1999). Antibodies against this biomarker are widely used for detection of circulating tumor cells (CTCs) in cutaneous melanoma and their overexpression has been correlated with the metastatic disease (Kawakami et al. 1997, Wang et al., 2001; Rodic et al., 2014). Moreover, MART-1 has been also studied as an immunotherapeutic agent to improve the immune response of patients with

melanoma (Mockey et al., 2007; Sioud et al., 2016). However, the use of MART-1 for the development of targeted nanoparticles to melanoma cells as drug delivery systems has not yet been conducted.

Based on these findings, the present study aimed to develop and characterize surface modified PBLG nanoparticles, including those functionalized with MART-1 monoclonal antibody conceived for intercepting melanoma CTCs within the blood stream, and evaluate their *in vitro* behavior by complement activation, cytotoxicity and cellular uptake assays.

Materials and methods

Materials

Dry N, N-dimethylformamide (DMF) and dry benzylamine were purchased from Acros Organics (Morris Plains, NJ, USA). Diethyl ether (DEE), methanol and tetrahydrofuran (THF) were purchased from Carlo Erba Reagents (Milan, Italy). Deuterated chloroform (CDCl₃), trifluoroacetic acid (TFA), Rhodamine B; 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), trans-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenylidene] malononitrile (DCTB), (3-[4,5-dimethylthiazol-2-yl]-2,5 CF₃COOK (Potassium trifluoroacetate), diphenyl tetrazolium bromide) (MTT), Dulbecco's phosphate buffered saline (PBS), Dulbecco's Modified Eagle's Medium - high glucose (DMEM), penicillin (100 U/ml), streptomycin (100 µg/ml), trypsin, glutamine (2µM), HABA avidin reagent and D-biotin-99% (TCL) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). N-carboxyanhydride of γbenzyl-l-glutamate (y-BLG-NCA) was purchased from ISOCHEM-SNPE (Paris, France). The α -methoxy- ω -amino-poly (ethylene glycol) (MeO-PEG-NH₂) Mw = 5 000 g.mol⁻¹, α -biotin- ω -amino-poly (ethylene glycol) (Bt-PEG-NH₂) Mw = 5 000 g.mol⁻¹ and α -methoxy- ω -
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carboxylic acid succinimidyl ester poly (ethylene glycol) (MeO-PEG₅-NHS) Mw = 5~000 g.mol⁻¹ from IRIS Biotech GMBH (Marktredwitz, DEU). PluronicW F68 (Lutrol F68) was provided by BASF (DEU).

The antibodies Melan-A/MART-1 Antibody (A103) [Alexa Fluor (R) 647] and Mouse IgG1 Kappa Light Chain Isotype Control (P3.6.2.8.1) [Biotin] were purchased from Novus Biologicals Europe (Abingdon, United Kingdom). Polyclonal anti-human C3 antibody raised in goat was purchased from Fitzgerald antibodies (Massachusetts, USA). The Goat anti-mouse IgG-HRP was purchased from Santa Cruz Biotechnology (California, USA). Streptavidin biotechnology grade was furnished by VWR International (Fontenay-sous-Bois, France). 100 kDa MWCO EMD Millipore Amicon[™] Ultra-0.5 centrifugal filters were obtained from Merck Millipore (Darmstadt, DEU). Water was purified by reverse osmosis (MilliQ, Millipore, USA).

B16-GFP melanoma cells were provided by Dr. Giorgia Egidy Maskos (INRA, UMR955, Maisons-Alfort, France). Human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (ATCC).

Methods

Synthesis of PBLG derivatives

Poly(γ -benzyl-L-glutamate)-benzylamine (PBLG-Bz), Poly(γ -benzyl-L-glutamate)poly(ethylene glycol) (PBLG-PEG) and poly(γ -benzyl-L-glutamate)-poly(ethylene glycol)biotin (PBLG-PEG-Bt) were synthesized by anionic ring opening polymerization (ROP) as previously described (Martínez-Barbosa et al., 2007). Briefly, the PBLG-Bz, PBLG-PEG and PBLG-PEG-Bt were prepared by the ROP of γ -benzyl-L-glutamate N-carboxyanhydride (γ -BLG-NCA) initiated by benzylamine (Bz), MeO-PEG-NH₂ and Bt-PEG-NH₂, respectively, in DMF. Firstly, γ -BLG-NCA (n mM) was weighed under inert atmosphere in an argon-purged three necks round-bottomed flask. The γ -BLG-NCA was dissolved in DMF at a concentration of 0.5 M and it was placed in a cold system, silica gel guard and a bubble detector. After 10 min of mechanical stirring at 30°C the argon flux was stopped. The initiator was added with an argon-purged syringe. Immediately after the addition of the initiator, CO₂ bubbles were observed. Absence of γ -BLG-NCA auto-polymerization was confirmed by infrared spectroscopy before addition of the initiator. The reaction evolution was controlled by infrared spectroscopy, by following the disappearance of characteristic NCA bands and the appearance of those of PBLG. Polymers were obtained by precipitation in an excess of cold DEE. The precipitate was filtrated and washed three times with methanol, in order to eliminate the excess of unreacted PEG. The polymers were finally washed with DEE and dried under vacuum at room temperature for at least 12 h. A second precipitation, purification and drying procedure were performed for all polymers.

Synthesis of PBLG-Rhodamine

PBLG-Rhodamine was synthesized using carbodiimide chemistry approach. Briefly, 2 g of PBLG-Bz and 0.02 g of EDC were weighed in an argon-purged three necks roundbottomed flask and dissolved in DMF. The solution was stirring for 10 min at room temperature with argon flux. Next, a solution of rhodamine B 0.05 M in DMF was added with an argonpurged syringe. The reaction mixture was then left in the darkness for 24 h. The precipitation, purification and drying procedures were carried out as described above. The polymer was washed several times with methanol in order to eliminate the excess of unreacted Rhodamine

Β.





Characterization of PBLG derivatives

Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FT-IR) spectra was carried out to analyze γ-BLG-NCA auto-polymerization and to follow the end of polymerization reaction using a FT-IR spectrophotometer (Perkin Elmer, USA).

Proton Nuclear Magnetic Resonance

Proton nuclear magnetic resonance (¹H NMR) spectra of the PBLG derivatives were recorded using a B-ACS 60 apparatus (Bruker, DEU) operating at 300 MHz. Samples were measured in CDCl₃ and 15% of TFA. For PBLG derivatives containing PEG as initiator, the molar mass of the polymer could be determined by the integration of the benzyl protons belonging to the PBLG block (7.26 (br, s, 5H, Ph–) or 5.04 (br, s, 2H, –CH₂–benzyl)), and the ethylene protons of the PEG block (3.65 (br, s, 4H,–OCH₂–CH₂O–)) considering the molar mass of the initiator.

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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses were performed using an UltrafleXtreme mass spectrometer (Bruker Daltonics, Bremen, DEU). Acquisitions were performed in linear positive ion mode. The laser intensity was set just above the ion generation threshold to obtain peaks with the highest possible signal-to-noise (S/N) ratio without significant peak broadening. All data were processed using the program Flex Analysis (Bruker Daltonics, Bremen, DEU). DCTB was used as the matrix for MALDI-TOF MS experiments and CF₃COOK was used as a cationizing agent.

Nanoparticles preparation

Preparation of self-assembled nanoparticles

Labeled and unlabeled nanoparticles were prepared with PBLG derivatives following a nanoprecipitation method previously described (de Miguel et al., 2014) for morphology and *in vitro* behavior evaluations. Briefly, pure (PBLG, PBLG-Rhod) or mixtures of PBLG derivatives (PBLG/PBLG-Rhod, PBLG-PEG/PBLG, PBLG-PEG/PBLG-Rhod, PBLG-PEG-Bt/PBLG and PBLG-PEG-Bt/PBLG-Rhod) with a polymer percentage ratio of 90:10 were dissolved in THF at 30°C during 18h. The polymer solution was added dropwise to a 0.125% poloxamer solution under magnetic stirring (700 rpm) for around 5 minutes. Solvents were evaporated under vacuum in the rotavapor at 40°C. The nanoparticle dispersions were filtered in ultracentrifugal filters (AmiconTM100 kDa) and stored at 4 °C for further use. The fluorescence of PBLG-Rhod (100%) and PBLG/PBLG-Rhod (10%) nanoparticles was verified in different times (3 and 6 h) in B16-GFP and HUVECs cells (5 x 10^4 cells/well) at the concentration 100 µg/mL by flow cytometry (MoFlo XDP, Beckman Coutler). Data were expressed as fold increase of mean relative fluorescence over negative control (cells without treatment).

The functionality of biotin on the surface of PBLG-PEG-Bt/PBLG nanoparticles was evaluated using the HABA/Avidin colorimetric method (Qi et al., 2004) The HABA dye binds specifically to avidin to produce a yellow-orange colored complex with absorption at 500 nm. Free biotin molecules displace the HABA dye causing an absorbance decrease. The amount of available biotin on the surface of the biotinylated nanoparticles was calculated and data are presented in units of nanomoles of biotin per mL of nanoparticles.

Preparation of the immunonanoparticles

Immunonanoparticles (PBLG-PEG-Bt-MART-1) were prepared using PBLG-PEG-Bt nanoparticles and the antibody MART-1 for targeting purpose. First, in order to obtain the theoretical nanoparticles/antibody ratio of 1:5, the approximate number of nanoparticles contained in 50 μ L were calculated based on the sphere equivalent diameters in volume from the transmission electronic microscopy (TEM) measurements of 130 nanoparticles (Eq. 1-3).

The theoretical number of biotin molecules per PBLG-PEG-Bt/PBLG-Rhod (90:10) nanoparticles was calculated (Eq. 4) based on the number of PBLG α -helices forming a nanoparticle, considering that each PBLG-PEG-Bt α -helix corresponds to a single biotin molecule (de Miguel et al., 2014). Nanoparticles were considered to be spherical and PBLG α -helices as rods, whose lengths were calculated by taking into account the projected segment

length of a single L-glutamate unit (= 0.15 nm), the number of residues of PBLG-PEG-Bt and PBLG-Rhod equal to 205 and 138, respectively, and the PBLG α -helices diameter of 1.6 nm as reported (Martínez-Barbosa et al., 2008)

Mass of 1 NP = density of PBLG ×
$$\left(\frac{4\pi (equivalent \ radius \ of \ NP)^3}{3}\right)$$
 (Eq. 1)

$$Equivalent \ Diameter = \sqrt[3]{Length * width^2}$$
(Eq. 2)

Number of
$$NP = \frac{Total \ polymer \ mass \ in \ 50\mu L}{mass \ of \ 1 \ NP}$$
 (Eq. 3)

$$N_{\alpha helix/np} = \frac{4r_{np}^3}{3(0.9 h_{\alpha helixA} r_{\alpha helixA}^2 + 0.1 h_{\alpha helixB} r_{\alpha helixB}^2)}$$
(Eq. 4)

where NP is nanoparticles, r_{np} is the mean radius of 130 nanoparticles measured by TEM, $r_{\alpha helixA}$ and $h_{\alpha helixA}$ are the radius and the length of PBLG-PEG-Bt helices, and $r_{B\alpha helix}$, $h_{B\alpha helix}$ are those of PBLG-Rhod helices, respectively.

Posteriorly, to insure an efficient antibody coupling and to avoid nanoparticle aggregation, a two-step immunonanoparticle formation was carried out. First, the complex antibodies-streptavidin was obtained using biotinylated antibody associated with an excess of streptavidin solution (1 mg/mL) during 2 h. After unreacted streptavidin was removed by PBS washing three times using ultracentrifugal filters (AmiconTM100 kDa). Next, MART-1-Streptavidin diluted in 50 µL of water was incubated with 50 µL of nanoparticles (1.5 mg/mL)

and left overnight on a rotating wheel at 4°C for conjugation reaction. Immunonanoparticles Université Paris-Saclay Université Paris Sud/ Institut Galien Paris Sud (CNRS UMR 8612), 5 rue Jean-Baptiste Clément, 92290 Châtenay-Malabry, France

of biotinylated IgG1-streptavidin was obtained as described above and used as nonspecific control.

Physicochemical and biological characterization of nanoparticles

Particle size, shape and surface charge

The size and shape of nanoparticles were analyzed by dynamic light scattering (DLS) and transmission electronic microscopy (TEM). The hydrodynamic diameter of nanoparticles was determined at 25 °C by with a Zetasizer 4 (Malvern, UK), operating at a fixed angle of 173°. For size measurements, 150 μ L of each sample was diluted in 850 μ L of deionized water (MilliQ, Millipore, USA). The temperature was allowed to equilibrate 5 min before measurement. Results are given as mean hydrodynamic diameter of the nanoparticles obtained from at least three measurements on three different preparations of nanoparticles.

TEM images were acquired with a JEM-1400 microscopy (JEOL, Japan), operating under a 120 kV accelerating voltage. The image analysis was carried out using Image $J^{(B)}$ software. A sample of 3 µL nanoparticles was placed on a copper grill covered with formvarcarbon film (400 mesh) and stained by phosphotungstic acid (1%) for 30 s. The mean size of 130 nanoparticles, from different images, was obtained by TEM. The width (smallest dimension) and the length (longest dimension) of individual nanoparticles taken from a series of microphotographs were measured.

The aspect ratio (Γ) of nanoparticles, which describes the shape of nanoparticles and how elongated they are, was calculated from TEM images using Equation 5.

Aspect Ratio
$$(\Gamma) = \frac{Length}{Width}$$
 (Eq. 5)

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Surface charge of nanoparticles was evaluated by zeta potential measurements (Zetasizer 4, Malvern, UK). For analysis, an aliquot of 500 μ L of nanoparticles was diluted in 500 μ L of NaCl/1 mM and data of zeta potential were expressed in millivolts.

Specific recognition of MART-1-coupled nanoparticles

To assess the MART-1 coupling on the surface of nanoparticles, *Western blotting* technique was used. Aliquots of coupled and uncoupled immunonanoparticles (with or without streptavidin) were centrifuged (16000 g for 40 min) and 10 μ L of the supernatant was denatured with 5% β-mercapto-ethanol in Laemmli buffer (20 μ L) boiled at 95 °C for 5 min. Non-denatured samples of immunonanoparticles (10 μ L) were diluted at Laemmil buffer in absence of β-mercapto-ethanol and without heating. Next, samples were loaded to a 4 to 15% gradient Mini-PROTEAN[®] TGXTM precast protein gels (Bio-Rad) and migrated at 200 V for 40 min. The migrated proteins were then transferred to a nitrocellulose membrane for 45 min at 100 V. The membrane was blocked in 5% nonfat milk in PBS–Tween (0.1%) for 1 h and incubated overnight at 4°C with a goat anti-mouse IgG-HRP diluted to 1/3000. Proteins were visualized with an enhanced chemoluminescent system (GE Healthcare, Velizy-Villacoublay, France).

Complement activation

The method of serial multiple crossed 2D immunoelectrophoresis was used for analyzing the complement C3 activation of nanoparticles (Coty et al., 2016). The analyses were carried out using nanoparticles presenting surface area ranging from 250 to 4000 cm²/mL or nanoparticles containing the same polymer concentration (1.5 mg/mL), corresponding to different surface areas ranging from 400 to 1875 cm²/mL. Briefly, nanoparticles samples (50 μ L) were incubated with Human serum (25 μ L) diluted in Veronal buffer saline (VBS2+) (25

 μ L) prepared as previous described (Kazatchkine et al., 1985). Samples were incubated for 1 h at 37°C, cooled at 4°C and analyzed in Agarose gel 1% containing anti-C3 antibody. The gel was prepared in Tricine buffer (Calcium Lactate 1 mM, TRIS 63 mM, Tricine 27 mM, in MilliQ[®] water, pH 8.6) and then casted on a Gel-FixTM film. Bands were cutted and then filled with 1% agarose gel prepared in Tricine buffer for the 1st dimension. Wells were formed at the same time using a homemade comb. The gel was placed in Multiphor II electrophoresis system (GE Healthcare, Velizy-Villacoublay, France) and wells were filled with 2.5 µL of samples. The samples were then subjected to the first-dimension electrophoresis (600 V, 16 mA, 100 W), freely migrating in the agarose gel band according to their molecular weight. Then, the gel was turned by 90° for the second dimension corresponding to the rocket immunoelectrophoresis (500 V, 12 mA, 100 W), for 3 h 30 min. Gels were dried, at room temperature, and stained with Coomassie blue to reveal the presence of proteins.

The gels were numerically scanned and area under peaks was integrated using ImageJ[®] 2.5 software. The first peak on the left side was recognized as native C3, whereas the second peak from the left was attributed to protein fragments derived from C3 cleavage (C3b). The complement activation factor (CAF) was calculated regarding the ratio of peak surface of cleaved protein C3b over the sum of the total peak surface of C3 native and fragments and also removing the natural activation (control) during the performance of the technique, according to the follow equations (Eq. 6-8).

Activation_{sample}(%) =
$$\left[\left(\frac{C3b_{sample}}{C3_{sample} + C3b_{sample}} \right) \times 100 \right]$$
 (Eq. 6)

Activation_{control}(%) =
$$\left[\left(\frac{C3b_{control}}{C3_{control} + C3b_{control}} \right) \times 100 \right]$$
 (Eq. 7)

$$CAF (\%) = \left[\left(\frac{Activation_{sample} - Activation_{control}}{100 - Activation_{control}} \right) \times 100 \right]$$
(Eq. 8)

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Receptor expression and cytotoxicity of the nanoparticles

First, the MART-1 expression in B16-GFP cells was verified. Cells were incubated with three different concentrations (8, 16 and 24 μ g/mL) of Melan-A/MART-1 Antibody (A103) [Alexa Fluor (R) 647] at 4 °C on ice in dark, for 1 h. After three PBS washings, MART-1 recognition was revealed using flow cytometry (BD FACSCalibur; BDbiosciences, Le Pont de Claix, France). HUVEC cells, MART-1 receptor-negative, are used as negative control.

The cytotoxicity of nanoparticles was assessed in B16-GFP cells and HUVECs. The cells were cultured in DMEM medium (pH 7.4 = 3.7 g/L NaHCO₃) containing 10% of bovine fetal serum, penicillin (100 unit/mL) and streptomycin (100 μ g/mL). The HUVECs growth medium was additionally supplemented with 2 mM of L-glutamine. Cells at logarithmic growth phase (3 x 10⁵ cell/mL), between ten and fiftieth passages, were seeded in 96-well culture plates at 10³ cell/well and incubated at 37°C for 24 h, 5% CO₂ and 95 °C humidity. Next, 100 μ L of nanoparticles, with polymer concentrations ranging from 1 to 500 μ g/mL, were added to each well and allowed to grow for 24 h. After incubation with 20 μ L of MTT (0.5 mg/mL in PBS) for 2 h, the supernatants were removed and 200 μ L of DMSO was added to solubilize the formazan crystals formed by viable cells. Viable cells were quantified by recording the UV absorbance at 570 nm using a plate reader multi-well scanning spectrophotometer (MRX II, DYNEX Technologies, Chanilly, USA). Untreated cells were used as control and assays carried out in triplicate. The IC₅₀, concentration that inhibits 50% of cells proliferation, was calculated by non-linear regression analysis with variable Hill slope given by parameter '*p*' using Origin 8.5.

The specificity of PBLG-PEG-Bt-MART-1 nanoparticles for B16-GFP cells (5 x 10^4 cells/well) was evaluated at 3 and 6 h in the concentration 100 µg/mL by flow cytometry (MoFlo XDP, Beckman Coutler). HUVECs cells were used as control, due to the absence of MART-1 receptor. Untreated cells were used as control and assays carried out in duplicate. Data of 10.000 events were analyzed per sample and the results were expressed as fold increase of mean relative fluorescence over negative control (cells without treatment).

Results and discussion

Synthesis and characterization of PBLG derivatives

PBLG derivatives were obtained and characterized with yield higher than 80% (Table 1). The polymerizations were followed by the disappearance of absorption bands in FTIR spectra of the anhydride ring at 1855 cm⁻¹ and 1787 cm⁻¹ of C₅=O and C₂=O, respectively, which indicated the end of the reaction (Fig. 2).

The amide absorption bands in the FT-IR spectra are used to characterize the polypeptide conformation (Fig. 3). For a polypeptide in an α -helix conformation, the amide I and the amide II bands are located at 1656 cm⁻¹ and 1548 cm⁻¹, respectively (Fontaine et al., 2001; Martínez-Barbosa et al., 2007; Segura-Sánchez et al., 2010) and for a β -conformation, the amide I and amide II bands are located at 1630 cm⁻¹ and 1536 cm⁻¹, respectively. Furthermore, an absorption band near to 1260 cm⁻¹ corresponding to the amide III band is observed in the FT-IR spectrum of a polypeptide in a α -helix conformation as previously reported (Martínez-Barbosa et al., 2007).

Polymers	Dpnt	γ-BLG-	[M]o	[I]o	Reaction	Yield	Molar
		NCA	$(mol L^{-1})$	$(mol L^{-1})$	time	(%)	mass
		(mmol)			(days)		$(g mol^{-1})$
PBLG-Bz	160	13.8	0.5	0.009	3	82	45 000 ⁽²⁾
PBI G-PFG	160	15.2	0.025	0 009	6	80	41 000 ⁽¹⁾
I DEG I EG	100	13.2	0.025	0.007	0	00	41 000
PBLG-PEG-Bt	160	15.2	0.025	0.009	6	80	50 000 (1)
							(2)
PBLG-Rhod	-	-	-	-	1	86	30 193(2)

Table 1. Characteristics of PBLG derivatives.

[M]o and [I]o are the initial concentration of γ -BLG-NCA and initiator, respectively. Dpnt indicates the theoretical values of each degree of polymerization. Polymers are denominated as follows: PBLG is followed by the name of the initiator (Bz: Benzylamine, PEG: poly (ethylene glycol) Molar mass was determined by NMR (1) and MALDI-TOF MS (2).

¹H NMR and MALDI-TOF MS techniques were used for determining the molar massfor each polymer (Table 1). The molar mass of PEGylated polymers was determined by the ratio between the peak intensities of methylene protons of PEG chain (OCH₂CH₂) and benzyl protons of PBLG chain (COOCH₂C₆H₅) (Fig.4). Due to the overlapping of the peaks of polymers without PEG in the NMR spectra, the determination of molar mass it was not possible using this technique. Thus, MALDI-TOF MS technique was used to overcome this problematic (Fig. 5a and b). All properties of PGLB derivatives are in agreement in our previous studies (Martínez-Barbosa et al., 2007; Segura-Sánchez et al., 2010).



Figure 2. FT-IR spectra of PBLG-Bz polymerization film recorded at 0 min (a), 58 min (b), 139 min (c), 277 min (d) and 570 min (e) of reaction time.



Figure 3. FT-IR spectra of PBLG derivatives: PBLG-Bz (I), PBLG-PEG (II), PBLG-PEG-Bt

(III) and PBLG-Rhod (IV).

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Figure 4. ¹H RMN spectra of PBLG derivates in CDCl₃: PBLG-Bz (I), PBLG-Rhod (II), PBLG-PEG-Bt (III) and PBLG-PEG (IV).

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Figure 5. MALDI-TOF MS spectra of PBLG-Rhod (a) and PBLG-Bz (b).

_Physicochemical and biological characterization of nanoparticles

Particle size, shape and surface charge

The nanoparticles were obtained from one or the mixture of two different PBLG derivatives following the nanoprecipitation method. The nanoparticles sizes were characterized by DLS and TEM and have a particle size between 20 to 100 nm with a narrow size distribution (PDI < 0.2) (Table 2). A 2-fold difference between the size measured by DLS and TEM was verified for all pegylated nanoparticles. These results can be explained by the presence of PEG chains on the nanoparticle surface, which increase the hydrodynamic radius measured by DLS. Saville and coworkers (2013) demonstrated that the hydrodynamic size determined by DLS of nanoparticles is directly proportional to molar mass of PEG polymers. On the other hand, the nanoparticles size of PBLG-Bz, PBLG-Rhod and PBLG-Bz/PBLG-Rhod measured by TEM presented similar size when compared with those obtained from TEM microphotographs, mainly due to the absence of PEG corona.

Regarding to the nanoparticles conjugated with antibodies (PBLG-PEG-Bt-MART-1/PBLG-Rhod and PBLG-PEG-Bt-IgG/PBLG-Rhod), it was observed an increase of the hydrodynamic size measured by DLS when compared to unmodified PBLG-PEG-Bt nanoparticles. This result indicates the presence of antibody moieties on the surface of nanoparticles, increasing the thickness of hydration layer. In addition, TEM micrograph demonstrated maintenance of the size and morphological aspects of PBLG-PEG-Bt nanoparticles after the antibody conjugation, indicating that particles are stable after surface modification. Equivalent results were observed in other studies with polymeric nanoparticles and antibodies conjugation (Chen et al., 2013; Ramon et al., 2013).



Related to the shape, it was observed that nanoparticles of PBLG-Bz, PBLG-Rhod, PBLG-Bz/PBLG-Rhod and PBLG-PEG/PBLG-Rhod exhibited a slightly higher aspect ratio ($\Gamma \approx 1.4$ to 1.6) with an ellipsoidal shape (Fig. 6 a, b, c and e). On the other hand, all the nanoparticles containing the PBLG-PEG-Bt presented rather spherical morphology ($\Gamma \approx 1.2$) (Fig. 6 d, f, g, h and i). Comparable results were found by Segura-Sanchez and coworkers (2010) for nanoparticles consisted of PBLG-Bz or PBLG-PEG-Bt. Studies verified that elongated nanoparticles can present a higher non-specific cellular internalization compared to the spherical particles mainly due to the large surface areas that facilitates particle-cell interactions (Gratton et al., 2008; Huang et al., 2010; Toy et al., 2014). However, not only the shape of nanoparticles can influence their cellular internalization rates, but specially their surface characteristics, as the presence of specific ligands that can be modified to improve and drive cellular uptake (Bertrand et al., 2014).

Nanoparticles presented a negative surface charge with zeta potential varying from -30 mV for PBLG to -3 mV for PBLG-PEG-Bt-MART-1 nanoparticles, suggesting a successful coating surface of nanoparticles (Table 2). As expected, the nanoparticles containing the neutral PEG-corona also demonstrated an increase of the zeta potential value (Table 2). The amphiphilic characteristic of the block copolymers PBLG-PEG and PBLG-PEG-Bt would be arrange the hydrophobic PBLG blocks at the core of nanoparticles, while the hydrophilic block containing PEG could be orientated on the surface of nanoparticles, forming a PEG-corona (Martínez-Barbosa et al., 2009; Segura-Sánchez et al., 2010).



In addition, the coupling of antibodies on PBLG-PEG-Bt nanoparticles increased their zeta potential when compared to uncoupled PBLG-PEG-Bt. As observed in another studies related to nanoparticles and antibody conjugation, this result is also an indicative of a successful modification of the surface of nanoparticles, in addition to the increase in particle hydrodynamic radius (Barua et al., 2013; Chen et al., 2013).

Nanoparticles	DLS mean size ± SD (nm)	PDI	TEM mean size ± SD (nm)	Aspect ratio	ζ potential \pm SD (mV)
PBLG	70 ± 1.3	0.17	80 ± 19.0	1.6	-30 ± 0.7
PBLG-Rhod	84 ± 1.6	0.07	97 ± 5.5	1.5	-14 ± 2.5
PBLG/PBLG-Rhod	69 ± 0.3	0.09	54 ± 15.0	1.4	-16 ± 0.2
PBLG-PEG/PBLG	44 ± 0.4	0.12	22 ± 3.2	1.3	-10 ± 1.3
PBLG-PEG/PBLG Rhod	51 ± 0.2	0.19	28 ± 4.0	1.4	-14 ± 1.5
PBLG-PEG-Bt/PBLG	54 ± 0.2	0.17	20 ± 1.5	1.2	-15 ± 0.5
PBLG-PEG-Bt/PBLG Rhod	58 ± 0.2	0.17	34 ± 8.2	1.2	-14 ± 3.8
PBLG-PEG-Bt-MART-1/PBLG Rhod	76 ± 0.6	0.20	34 ± 6.0	1.2	-3.0 ± 0.5
PBLG-PEG-Bt-IgG/PBLG Rhod	74.5 ± 0.6	0.20	33 ± 5.5	1.2	-3.2 ± 0.3

Table 2. Morphological characteristics and zeta potential of nanoparticles.

DLS: dynamic light scattering, PDI: polydispersity index, TEM: transmission electronic microscopy, SD: standard deviation, ζ potential: zeta potential.



Figure 6. TEM photographs of nanoparticles obtained from the following PBLG derivates. Magnification 20000 ×. PBLG-Bz (a), PBLG-Rhod (b), PBLG/PBLG-Rhod (c), PBLG-PEG/PBLG (d), PBLG-PEG/PBLG-Rhod (e), PBLG-PEG-Bt/PBLG (f), PBLG-PEG-Bt-Bt/PBLG-Rhod (g), PBLG-PEG-Bt-MART-1/PBLG-Rhod (h) and PBLG-PEG-Bt-IgG/PBLG-Rhod (i).

The fluorescence of PBLG-Rhod and PBLG/PBLG-Rhod nanoparticles was demonstrated by the cellular uptake assay carried out in B16-GFP and HUVECs cells (Fig. 7). The mean fold changes in fluorescence intensity over control for PBLG-Rhod nanoparticles varying from 1.4 to 1.9 for HUVECs cells and 1.5 to 2.3 for B16-GFP cells. In addition, when the cells were treated with PBLG/PBLG-Rhod the mean fold changes over control approximately 1.2 for HUVECs cells, and 1.1 to 1.4 for B16-GFP cells. These results

demonstrated a weak red fluorescence signal detected at cellular uptake experiments, after incubation with PBLG-Rhod and PBLG/PBLG-Rhod nanoparticles, which can be attributed to the low fluorescence intensity of the obtained PBLG-Rhod derivative. Despite of the low fluorescence observed for PBLG-Rhod nanoparticles, these nanosystems can be used on GFP expressing cells as an alternative, especially due to its relatively low cost when compared with other fluorescent labels, such as Alexa Fluor 495. In this way, this remark must be considered for further studies combining fluorescent and targeted nanoparticles, in order to obtain an optimized fluorescent PBLG derivative.



Figure 7. Cellular uptake of PBLG-Rhod (100%) and PBLG/PBLG-Rhod (10%) nanoparticles by B16-GFP and HUVECs cells. Data were expressed as fold increase over control (mean ± SD).

Functionality of the biotin grafted onto PBLG-PEG-Bt nanoparticles

The conjugation of the antibody on the surface of PBLG-PEG-Bt nanoparticles via biotin–avidin complexation was first suggested by Segura-Sanchez and coworkers (2010). This methodology to bind antibodies on the surface of nanoparticles is based on the well-described strong affinity of biotin for avidin molecule (Wright et al., 1947). The percentage of biotin available and accessible on the surface of nanoparticles prepared with PBLG-PEG-Bt was approximately 56%, corresponding a theoretical number of 190 accessible biotin molecules per nanoparticle, given that each individual nanoparticle is formed by approximately 340 PBLG- α helix chains. These results can be justified by hydrophilicity, flexibility and length (MW= 5000 g/mol) of the PEG-Bt chains, which favors biotin molecules towards on the surface of nanoparticles and their availability for avidin binding (Segura-Sanchez et al., 2010). As expected, biotin was not detected in PBLG-PEG/PBLG nanoparticles.

Specific recognition of MART-1-coupled nanoparticles

The MART-1 was chosen as a biomarker model to improve the receptor-mediated endocytosis and intracellular drug delivery to melanoma cancer cells. As show in the Figure 8, when nanoparticles were incubated with streptavidin-antibody complex, no signal of uncoupled antibodies was detected. These results demonstrated that the binding of the antibody on the surface of nanoparticles occurs mediated by streptavidin, meanwhile in the absence of streptavidin, the remaining free antibodies were observed. Similar results were found by Ramon and coworkers (2013).

The antibody binding on the surface of nanoparticles was also evidenced by the different migration profiles in western blot analysis of non-denatured MART-1-nanoparticles

in presence or in absence of streptavidin. The broader migration profile detected for antibody conjugated to nanoparticles when compared to uncoupled antibodies. Can be attributed to the different morphological and surface characteristics of the MART-1-nanoparticles, which provides a heterogeneous migration profile through the electrophoresis gel. In addition, the western blot analysis of the non-denatured antibody-streptavidin complex showed a different migration profile when compared to the antibody conjugated to nanoparticles. As shown in Figure 8 the antibody-streptavidin complex migrates above 220 kDa, corresponding approximately to the molar mass of streptavidin (55 kDa) plus antibody (150 kDa). These results indicate the ability of PBLG-PEG-Bt nanoparticles to bind with biotinylated antibodies through the formation of biotin-streptavidin complexes, allowing the formation of immunonanoparticles.



Figure 8. Western blot analysis of the remaining free antibodies found in supernatant of biotinylated nanoparticles and the migration profile of non-denatured nanoparticles containing Mart-1 antibody (a) and control antibody (b) after incubation with (+) or without (-) streptavidin. The Western blot analysis also demonstrated the migration profile of the complex antibody-streptavidin (mAb+Strep) non-denatured and denatured. The theoretical ratios of the number of nanoparticles over the number of antibody molecules were 1:0 and 1:5.

Complement activation

The complement system is a major component of the innate immune response against foreign particles and bodies that have the potential to be infectious or pathogenic (Noris & Remuzzi, 2013). A proper biodistribution and long blood lifetime of intravenously administered nanoparticles depends on the extent of their non-specific recognition and opsonization by complement system (Bertholon et al., 2006). It is also well reported that different size and surface properties of nanoparticles can directly influence their interactions with serum proteins and define their clearance from blood circulation (Moghimi et Szebeni, 2003; Lundqvist et al., 2008).

The complement component 3, commonly called C3 protein, is the major component of final pathway of complement cascade and is common to all three complement activation pathways (classical, lectin and alternative). The C3 convertase enzyme cleaves the C3 protein in fragments, C3a and C3b. The C3b is responsible for opsonization of foreign bodies, such as nanoparticles, through the covalent attachment to the particle surface, increasing the antibody response and facilitating foreign bodies clearance through the reticule endothelial system (Nilsson et al., 2007; Noris et Remuzzi, 2013). In this way, the evaluation of complement activation, independently of the pathway, can be measured by the cleavage of C3 protein. The results are represented by eletrophoregrams that generally depicts two peaks, the first related to the native C3 and the second related to the fragment C3b (Coty et al., 2016).

A prior analysis of the ability of PBLG and PBLG-PEG nanoparticles to activate the complement system was performed with samples containing similar surface areas ranging from 250 to 4000 cm²/mL (Fig. 9). As shown in Figure 9 the complement activation in all tested

concentrations were higher for PBLG nanoparticles when compared with PBLG-PEG nanoparticles. At the highest concentration, 4000 cm²/mL, the % of activation for PBLG was 26% against 11% for PBLG-PEG nanoparticles, which indicated the stealth capability of hydrophilic PEG corona.

In addition, we evaluated nanoparticles with different surface areas, ranging from 400 to 1875 cm²/mL, which can influence the activation of complement cascade, since the protein interactions depends on the available area of the nanoparticle (Noris et Remuzzi, 2013). The electrophoregrams and respective %CAFs for each tested nanoparticle are depicted in Figure 10. As observed, all the obtained CAF values were below 15%, whereas the positive control showed a CAF of 77%, demonstrating a low ability of these nanoparticles to activate the complement system when they are at the same polymer concentration (1.5 mg/mL) (Coty et al., 2016). In addition, the CAF for immunonanoparticles was lower than PBLG-PEG-Bt nanoparticles, indicate an increase in the furtiveness of this nanocarrier after surface modification. Nevertheless, as expected, the nanoparticles containing only PBLG at a tested surface area (S_A) of 470 cm²/mL or the mixture PBLG/PBLG-Rhod (S_A= 700 cm²/mL) demonstrated a higher %CAF compared to the nanoparticles containing PEG with higher surface areas, ranging from 1100-1875 cm²/mL. These findings are also consistent with the giving stealth properties of PEG hydrophilic corona (Owens et Peppas, 2006). Besides, nanoparticles did not activate the complement cascade even after their surface modification by coupling antibodies or after the addition of fluorescent PBLG-Rhod.



Figure 9. C3 activation by nanoparticles as a function of the concentration of PBLG and PBLG-PEG. The green line represents the spontaneous activation.

Positive Control	PBLG	PBLG/PBLG-Rhod	PBLG-Rhod	PBLG-PEG/PBLG
~			<u> </u>	A
CAF= 77%	CAF= 10.4 ± 1.3 %	CAF= 14.7 ± 3.3 %	CAF= 6.7 ± 0.5 %	CAF= 7.1 ± 0.03 %
Negative Control	PBLG-PEG/PBLG-Rhod	PBLG-PEG-Bt/PBLG	PBLG-PEG-Bt/PBLG-Rhod	PBLG-PEG-Bt-MART-1/PBLG-Rod
A	5	14	4	1
Spontaneous activation	CAF= 4.5 ± 0.4 %	CAF= 4.2 ± 0.4 %	CAF= 1.6 ± 0.6 %	CAF= -4.9 ± 0.02 %

Figure 10. Electrophoregram peaks of complement activation for different PBLG nanoparticles with the respective complement activation factor (% CAF).

Receptor Expression and Cytotoxicity of nanoparticles

The evaluation of MART-1 expression on B16-GFP-melanoma and on HUVECs cell lines was performed by flow cytometer analysis after labeling cells with anti-MART-1 antibody coupled with Alexa Fluor (R) 647 at three different concentrations. The results depicted in Figure 11 a and b show an approximately 25-fold shift of the mean fluorescence of B16-GFP melanoma cells after incubation with all tested antibody concentrations. No differences between the fluorescence mean was observed when the melanoma cells were incubated with 16 or 24 μ g/mL of MART-1 antibody, indicating a saturation of the MART-1 binding sites presented on the surface of B16-GFP melanoma cells. It was also demonstrated that approximately 57% of the B16-GFP melanoma cells are positive for MART-1 receptor (Fig. 11a). These results confirm that B16-GFP-melanoma cells present the MART-1 receptors. On the contrary, HUVECs cells did not exhibited a significant fluorescence after MART-1 antibody incubation, thus this lineage was used as a negative control, since they not express MART-1 receptors.



Figure 11. Evaluation of the MART-1 expression on B16-GFP cells and HUVECs cells by flow cytometer. Percentage of B16-GFP cells (MART-1 positive; upper right quadrant) and the number of viable cells shown at each time point (a). Data of fluorescence intensity are shown as mean fold over control as function of the antibody concentration (b).

The cell viability graphs for B16-GFP-melanoma cell line and HUVECs cells are depicted in Figures 12 and 13, respectively. In Fig. 12a the IC₅₀ values for PBLG and PBLG/PBLG-Rhod nanoparticles were 306 \pm 5.6 and 296 \pm 4.2 µg/mL, respectively. Regarding the non-fluorescent pegylated nanoparticles, IC₅₀ values of 313 \pm 4.5 and 320 \pm 3.3 µg/mL were verified for PBLG-PEG and PBLG-PEG-Bt, respectively in B16-GFP cells. However, the IC₅₀ values of fluorescent pegylated nanoparticles decreased to 240 \pm 2.3 and 184 \pm 4.4 µg/mL for PBLG-PEG and PBLG-PEG-Bt, respectively (Fig. 12b). In addition, the nanoparticles coupled with antibody–avidin complexes, PBLG-PEG-Bt-IgG (IC₅₀= 156 \pm 6 µg/mL) and PBLG-PEG-Bt-MART-1 (IC₅₀= 138 \pm 5.1 µg/mL) demonstrated an enhancement in in vitro cytotoxicity against B16-GFP melanoma cells compared to uncoupled PBLG-PEG-Bt nanoparticles (Fig. 12c).

Related to HUVECs, the IC₅₀ values for PBLG and PBLG/PBLG-Rhod were 236 ± 8.5 and $162 \pm 4.2 \ \mu g/mL$, respectively (Fig. 13a). In addition, the IC₅₀ values of pegylated nanoparticles varied from $138 \pm 1.3 \ \mu g/mL$ to $283 \pm 4.3 \ \mu g/mL$. Those finds suggest that the cytotoxicity profile of PBLG based nanoparticles can be affected by their composition and design of their surface. However, a significant decrease of B16-GFP and HUVECs cells viability treated with all nanoparticles formulations was only verified at concentrations above 200 $\mu g/mL$. Differently from some polymeric nanocarriers that need to be surface modified to enhancing its tolerance, for example, poly(isobutylcyanoacrylate) (PBICA) nanoparticles coated with polysaccharides (Chauvierre et al., 2007), the obtained PBLG based nanoparticles demonstrated to be well tolerated by cancerigens and normal cells.

Other studies with nanoparticles, polymersomes and micelles based on different PBLG copolymers, also observed a low cytotoxicity of these nanocarriers when tested in normal and

cancerous cells, at polymer concentrations varying from 1 to 500 μ g/mL (Du et al., 2010; Upadhyay et al., 2010; Thambi et al., 2011; Goñi-de-Cerio et al., 2013). In this way, the developed PBLG nanoparticles presented a low cytotoxicity in tested cells, suggesting that these nanoparticles can be suitable for anti-cancer drug delivery.

a)



Figure 12. B16-GFP cells viability percentage after treatment with PBLG-derived nanoparticles using the MTT assay. Error bars indicate the standard deviation.







Figure 13. HUVECs cells viability percentage after treatment with PBLG-derived nanoparticles using the MTT assay. Error bars indicate the standard deviation.

Cellular uptake of immunonanoparticles

As observed in Figure 14, the cellular uptake of PBLG-PEG-Bt-MART-1 nanoparticles by B16-GFP when compared to HUVECs cells was increased in 50% and 40% after 3 and 6 h of incubation, respectively. This result indicates a specificity of PBLG-PEG-Bt-MART-1 nanoparticles for the tested melanoma cells due to the presence of MART-1 receptor on the surface of B16-GFP cells. These results show that the B16-GFP cells could be recognized by PBLG-PEG-Bt-MART-1 nanoparticles containing only five molecules of MART-1 antibody coupled at their surface, which indicates a specificity of this target molecule for the tested melanoma cells due to the presence of MART-1 receptor on the surface of B16-GFP cells. Some studies have also shown that immunonanoparticles conjugated with antibodies in a ratio ranging from 2 to 10 antibodies per nanoparticle, can promote an effective cell-particle binding and trigger a receptor-mediated endocytosis (Funovics et al., 2004; Ho et al., 2009; García-Fernández et al., 2017). Similarly, Ramon and coworkers (2013) evaluated three different ratios of nanoparticles per antibody molecules (1:5; 1:10 and 1:20) and demonstrated that the ratio of five molecules of a specific antibody to Ewing's sarcoma, conjugated with PIBCA/chitosan nanoparticles, were sufficient to achieve cell recognition.

Ho and coworkers (2009) analyzed how the number of conjugated Herceptin antibodies per nanoparticle (1.9 to 9.4 antibodies per particle) influence on the cell-particle binding and elucidated if the binding events were monovalent or multivalent. The authors concluded that Herceptin immunonanoparticles (round shape, size of 80 nm) follow the theoretical behavior of monovalent binding and did not presented a threshold antibody density for binding but demonstrated that the cell-particle affinity increases linearly with increasing antibody conjugation density. In this way, the increase of the theoretical ratio number of nanoparticles/antibody molecules could also be a strategy to potentialize the specific capture of PBLG-PEG-Bt-MART-1 nanoparticles by melanoma cells that express MART-1 antigen, but would require an excess of antibody, which significantly increases cost of the formulation



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Figure 14. Cellular uptake of PBLG-PEG/PBLG-Rhod, PBLG-PEG-Bt/PBLG-Rhod and PBLG-PEG-Bt-MART-1/PBLG-Rhod nanoparticles by HUVECs (a) and B16-GFP (b) cells. Data were expressed as fold increase over control (mean \pm SD).

The high expression of MART-1 peptide in cytoplasmic organelles, especially in endoplasmic reticulum, makes this biomarker an important target for therapies that aim to delivery exogenous peptides to this organelle and increase the cross-presentation of antigens for the induction of adaptive immunity against tumor cells (Li et al., 2008; Sneh-Edri et al., 2011). Moreover, it has been reported that the treatment of melanoma patients with specific BRAF-inhibitors could increase the expression of MART-1 peptide, which can be used as an opportunity for rational combinations of systemic targeted therapy and immunotherapy for melanoma (Boni et al., 2010). In this way, the next step for this research would be to study the pathways of endocytosis of these nanoparticles and the cytoplasmic distribution of these carriers in melanoma cells for intracellular drug-delivery applications.

Conclusion

In this work, we obtained stealth, fluorescent label and site-specific nanoparticles based on PBLG derivatives in a nanoscale and homogeneous size. The furtiveness with PEG and targeting with biotin and MART-1 influenced on particle size, shape and surface charge of surface-modified nanoparticles prepared with PBLG derivatives. PBLG nanoparticles were used as a new platform to design targeted immunonanoparticles using MART-1 as a biomarker. The immunonanoparticles containing MART-1 were targeted to B16-GFP cells, not activate the complement system and present low cytotoxicity. In general, this research shed light to the optimization of important parameters on the formulation of PBLG nanoparticles, such as the fluorescence of the PBLG-derivatives and the ratio number of nanoparticles/antibody molecules, for the development of promising systems to carry anticancer drugs to be used in melanoma therapy.

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CHAPITRE V

GENERAL DISCUSION



GENERAL DISCUSSION

Introduction

The migration of cancer cells from the primary tumor and their establishment in secondary sites, gives rise to metastasis, which is concerning nearly 90% of cancer-related deaths (Gupta e Massagué, 2006). Considering that cancer is one of the leading causes of morbidity and mortality worldwide with approximately 14 million new cases and 8.2 million cancer related deaths in 2014, according with the report of the World Health Organization, the development of different approaches to improve the current strategies in cancer therapy in a broad sense are therefore highly desirable.

In the complex progression of metastasis, some cancer cells of the primary tumor will dissociate from the solid tumor, followed by the invasion of the extracellular matrix. After a successful invasion of the surrounding tissue microenvironment, tumor cells intravasate into the bloodstream and/or lymphatic flow. These cells exhibit a phenotypic modification called epithelial-mesenchymal transition (EMT). When they have reached the blood, these cells are generally referred as circulating tumor cells (CTCs). These cells (or discrete cells aggregates) are then distributed in the body and disseminated in distant organs. For instance, these cells can be arrested either in capillary beds or directly at blood vessels surface. Once in a favorable environment, the CTCs will marginate actively towards the endothelial walls and extravasate out of the bloodstream. Finally, they can diffuse, colonize an organ and form secondary tumors (Gupta et Massagué, 2006; Chaffer et Weinberg, 2011; Alizadeh *et al.*, 2014). The epithelial-mesenchymal transition (EMT) has a major role in tumor progression, been associated with the induction of invasive capacity and the formation of circulating tumor cells (CTCs). It has

been observed that the activation of the Snail, Slug, Twist proteins, among other molecules, could trigger EMT transition. Furthermore, the activation of EMT is not exclusively providing a fully metastasis potential but is also providing resistance to cell death, radioresistance, and chemoresistance, since the machinery of genes that is used plays a crucial role in embryonic development, wound healing and regeneration.

Compared to the large number of CTCs continuously escaping from a solid tumor, the process is highly inefficient, as only 0.01% of circulating tumor cell (CTCs) will form successful secondary tumors (Hong et Zhang, 2016). However, CTCs has an aggressive profile which is related to the selection that undergoes in heterogeneous cell subpopulations produced after their capacity to interact with the tumor stroma resulting in a release of signals that enhance tumor growth, angiogenesis, invasion, and metastasis (Jacob *et al.*, 2007; Bertazza *et al.*, 2008). Considering that CTCs can be released in early steps of primary tumor growth and could seed either the primary tumor or distant metastasis as observed by Norton and Massagué (Norton et Massague, 2006) and Kim and colleagues (Kim *et al.*, 2009), their detection in cancer patients is related to poor prognostic and survival as this evidence could explain also the relapse of tumor reseeding despite tumor removal.

Therefore, considering the theoretical and experimental data available on tumor spreading, the capture of CTCs and their elimination could be of interest when aiming to avoid or at least delay metastasis development. Further, when considering the highly differentiated biophysical properties of the different tissues in which the CTCs are migrating, the blood compartment can be favorably envisioned as a locus in which it could be simpler to capture and eliminate the CTCs during their migration process. In the present work, we focused on the hypothesis that polymeric nanoparticles could be suitable for this aim.

Since decades, nanoparticles have emerged to overpass the drawbacks of traditional therapies. From preventing drug degradation or enhance drug solubility to targeted applications, nanoparticles have become an interesting strategy to address specific aims in drug delivery (Kingsley *et al.*, 2006). In recent years, considering the great advances in material sciences, the design of nanoparticles and nanomedicines in general, the design of nanoparticles is becoming more rational as well as more precise. Main efforts are focused in conceiving highly customized sizes, shapes and molecular surface characteristics, to modulate their fate in the body (Duan et Li, 2013; Toy *et al.*, 2013; Deng et Changyou, 2016; Toy et Roy, 2016).

Within this framework, considering our hypothesis that CTCs need to be recognized and treated in the circulation, a major question arises. What are the optimal characteristics that will the nanoparticles able to find circulating tumor cells in the complex blood environment, especially considering that there are only few CTCs per billion of other blood components, including blood cells, proteins, exosomes, etc. For this issue, the phenomenon of the margination of the nanoparticles has been contemplated as a crucial parameter to enhance nanoparticle/CTC interaction probability. Margination phenomenon occurs when a particle that is under hydrodynamic forces in a flow, is deflected towards the walls. When considering a spherical particle, the forces are homogeneously distributed on the whole surface, giving rise to an homogeneous motion of the particles in the liquid. Thus, due to the hemodynamic flow, the particle remains preferentially located in the center of the flow. In contrast, forces are heterogeneously distributed on elongated particles, which generate both rotational and translational movements, allowing the nanoparticle to escape from the center of the flow towards the walls (Doshi *et al.*, 2010; Thompson *et al.*, 2013; Toy *et al.*, 2013; Ponchel et Cauchois, 2016). Therefore, if the particle could spontaneously marginate, it is expected that its capacity to explore a larger volume of fluid, could be more appropriate to 'sense' CTCs along the capillary's endothelium. Thus, the nanoparticles geometry appears to be an important requisite for this application. Obviously, the general architecture of the nanoparticles would comprise simultaneously a careful engineering of the surface for gaining in specific recognition of CTCs, while avoiding non-specific capture by cells in the vascular compartment.

The objective of this work was to design nanoparticles based on amphiphilic $poly(\gamma$ benzyl L-glutamate) (PBLG) copolymers. A set of di- and tri-block amphiphilic copolymers of PBLG has been produced which presents a variety of lengths and arrangement of hydrophilic PEG blocks and hydrophobic PBLG α -helix blocks. The selection of copolymers provides a range of nanoparticle architectures once prepared by a nanoprecipitation method.

Conception and synthesis of a mini library of PBLG derivates

In the exploration of biocompatible materials for drug delivery systems, synthetic polymers have rouse interest due to their potential to be synthesized with specific functionalities (Tiwari et Bellare, 2012). Among the variety of synthetic polypeptides, poly(γ -benzyl L-glutamate) (PBLG) is an attracting block. Belonging to the large family of poly(α -amino acids), it has drawn attention for biomedical applications due to the presence of a degradable amide in the polymer backbone and a carboxylic acid function on the lateral side chain, available after the hydrolysis of the lateral benzylic esters. In addition, after polymerization PBLG chains are ended by a reactive amine group, freely accessible for further modifications. In addition, since decades, PBLG it is considered as a solid candidate to form pharmaceutically acceptable nanoparticles due to the spontaneous self-assembly of these



copolymers in aqueous conditions (Anderson *et al.*, 1985; Martínez Barbosa *et al.*, 2007; Martinez Barbosa *et al.*, 2008; De Miguel *et al.*, 2014).

In this work, PBLG derivatives were synthesized following different approaches, firstly by ring-opening polymerization (ROP) of γ -benzyl-L-glutamate N-carboxyanhydride (BLG-NCA) initiated by amino-based initiators, using a slightly modified method described by Martinez and colleagues (Martínez Barbosa *et al.*, 2007), and then a chemical modification through carbodiimide crosslinker chemistry described by De Miguel and colleagues (De Miguel *et al.*, 2014). The mechanism described by Martinez and colleagues was a ROP polymerization using primary amines as initiators. This pathway has been selected because of the high reactivity of primary amino groups which are more reactive than the growing chain. The initiation is thus faster than the propagation and therefore, the degree of polymerization can be predicted by the ratio of NCA / initiator (Fontaine *et al.*, 2001).

Synthesis of PBLG di-block copolymers

A set of di-block copolymers PBLG₃₅-Bnz, PBLG₃₅-PEG₅, PBLG₄₅-PEG₅-biotin were synthetized according to protocols established by Martinez and colleagues (Martínez Barbosa *et al.*, 2007) and Segura-Sanchez and colleagues (Segura-Sánchez *et al.*, 2010). The polymers PBLG₃₅-Bz and PBLG₃₅-PEG-biotin were conceived using a PBLG hydrophobic block with a molar mass of 35 000 gmol⁻¹. Furthermore, for PBLG-PEG copolymers, two different molar mass were synthetized to be used in parallel as the base of the tri-block copolymer PEG₅-PBLG₇₀-PEG₅.

A PEG molar weight of 5000 Da was selected since it has been reported to confer stealth properties to the nanoparticles, via an appropriated reduction of seric proteins opsonization via steric hindrance (Gref *et al.*, 2000). Simultaneously, this molecular weight is low enough to make possible renal excretion of this non-degradable polymer.

Synthesis of PBLG tri-block copolymers

According to the literature, several tri-block copolymers have been proposed as a promising approach for nanomedicine applications. For example, Kim and colleagues (Kim *et al.*, 2012) formulated Herceptin conjugated Poly(caprolactone)–poly(ethylene glycol)–poly(caprolactone) (PCL-PEG-PCL) nanoparticles for cancer targeting and imaging, as the resultant nanoparticles were loaded with quantum dots. Another interesting application was proposed by Fu and colleagues (Fu *et al.*, 2011). The authors formulated gene-loaded poly(lactic acid)-poly(ethylene glycol)-poly(L-lysine) (PLA-PEG-PLL) nanoparticles as non-viral vectors to enhance gene transfections. According to their work, the polymer blocks were selected to obtain self-assembled nanoparticles. However, as indicated by the authors, the selection of PLL polymer was based on their capacity to interact with the phosphate groups of DNA. Thus, considering the current approaches in nanomedicine applications, the synthesis of tri-block copolymers was pursued.

A PEG₅-PBLG₇₀-PEG₅ tri-block copolymer was prepared in a two steps protocol. Firstly, PBLG₇₀-PEG₅ was synthetized and purified as a di-block copolymer. Once PBLG₇₀-PEG₅ was characterized, a carbodiimide reaction was performed to attach the second PEG chain, using MeO-PEG₅-NHS and the primary amine of PBLG chain. This approach had been previously carried out successfully in our group (De Miguel *et al.*, 2014). The selection of PBLG molar mass was based on the observations of Cauchois and colleagues (Cauchois *et al.*, 2013). They observed that an increase of the molar weight of PBLG chains resulted in nonspherically shaped nanoparticles. They determined that the aspect ratio of the nanoparticles was dependent on the length of the alpha helix rods, i.e. the molecular weight of PBLG chains. While determining the optimal length of the PBLG block, the hydrophobic/hydrophilic balance was also considered since it was a mandatory characteristic to ensure a correct self-assembling of the amphiphilic copolymers.

For preparing the tri-block PBLG₃₅-PEG₁₀-PBLG₃₅ and PBLG₇₀-PEG₁₀-PBLG₇₀ copolymers, a ROP polymerization was performed using a bis-amino poly (ethylene glycol) with a molar mass of 10 000 gmol⁻¹. As explained above, the primary amine is extremely more reactive than the growing chain. Therefore, it is predicted that the two primary amines react at equal time. Thus, both sides will have an identical length. In terms of PEG, the molar mass was chosen to provide the stealth property, as well as grant enough length for the arrangement of PBLG chains, once used to formulate nanoparticles in an aqueous medium.

Synthesis of fluorescent-labeled PBLG copolymers

Two fluorescent-labeled polymers were obtained by a carbodiimide chemistry approach from a preformed PBLG₃₅-Bz. Despite that rhodamine and cyanine 3 (Cy3) have a similar excitation/emission spectrum, Cy3 presented an advantageous stability in terms of time. It was found that the rhodamine-labeled copolymer lost the fluorescent intensity level with time despite appropriated preservation conditions. In addition, the Cy3-labeled copolymer was prepared to increase the fluorescence intensity requested for cytometry measurements, as well as in vivo imaging.

Preparation of multifunctional nanoparticles

In terms of polymeric nanoparticle preparation, several pathways have been proposed depending of the material characteristics (Tiwari et Bellare, 2012). First developed by Fessi and colleagues in 1989, nanoprecipitation method allows to form nanoparticles by spontaneous self-assembly phenomenon (Fessi *et al.*, 1989). Indeed, by controlling the characteristics of the preformed polymer, it is possible to orientate the nanoparticle architecture (Letchford et Burt, 2007).

Thus, the preparation of PBLG-based nanoparticles was achieved by nanoprecipitation method described elsewhere (Martinez Barbosa *et al.*, 2008; Özcan *et al.*, 2011; De Miguel *et al.*, 2014). Two sets of PBLG nanoparticles were prepared, the first set was precipitated in 0.125% w/v Poloxamer solution and the second set was prepared by direct nanoprecipitation in Milli-Q water.

In this work, due to the disposition of PEG chains, some systems could give rise to anisotropic surfaces (one part of the surface being hydrophilic, while the other was hydrophobic). Thus, poloxamer was introduced with the aim of separate the possible combination of the shape effect and the hydrohilic/hydrophobic balance of the surface. Poloxamer is a tri-block copolymer in ABA conformation, formed by a central hydrophobic chain of polypropylene oxide (PPO) flanked by two hydrophilic chains of polyethylene oxide (PEO) (Santander-Ortega *et al.*, 2006). The PPO central block, can be strongly adsorbed onto hydrophobic surfaces of the nanoparticles, leading to the masking of the surface by hydrophilic PEO chains to form an hydrophilic layer equivalent to the one obtained by the presence of PEGs at the surface (Alexandridis e Alan Hatton, 1995; De Sousa Delgado *et al.*, 2001).

Therefore, all systems prepared with poloxamer presented a complete hydrophilic layer in the surface, while the nanoparticles presented in water exhibited anisotropic surfaces.

Preparation of di-block PBLG-based nanoparticles

Nanoparticles prepared from PBLG₃₅-Bz, PBLG₃₅-PEG₅, PBLG₃₅-PEG₅-biotin presented similar characteristics to those previously reported in our group (Martínez Barbosa *et al.*, 2007; Segura-Sánchez *et al.*, 2010). However, as expected and according to Cauchois and colleagues, the nanoparticles were slightly more spherical, which could be related to the length of the PBLG bloc. It was also observed that in the presence of poloxamer, the nanoparticles exhibit a smaller size. Such a behavior that has been also reported in the literature (Yan *et al.*, 2010). An extended analysis of the characteristics of the nanoparticles have been performed in chapter 2.

Preparation of tri-block PBLG-based nanoparticles

As observed in the literature, nanoparticles prepared from tri-block copolymers present a spherical morphology as observed in the work of Fu and colleagues (Fu et al., 2011) and Kim and colleagues (Kim *et al.*, 2012), described above. Interestingly, two of our tri-block based nanoparticles presented a non-spherical morphology.

Considering the tri-block systems prepared in this work, PEG₅-PBLG₇₀-PEG₅-based nanoparticles presented the most well-defined elongated morphology, followed by PBLG₇₀-PEG₁₀-PBLG₇₀ nanoparticles. Despite the similarity of PBLG₇₀-PEG₁₀-PBLG₇₀ and PBLG₃₅-PEG₁₀-PBLG₃₅ copolymers, a spherical morphology was obtained for the tri-block PBLG₃₅-PEG₁₀-PBLG₃₅.

The differences in the morphology within the tri-block nanoparticles was highly related to the length of PBLG chains. This was evident when PEG₅-PBLG₇₀-PEG₅ nanoparticles are compared to the 'most similar' system, PBLG₃₅-PEG₅ nanoparticles. In PEG₅-PBLG₇₀-PEG₅ nanoparticles with an aspect ratio of 2.6, the PBLG segment is about of ~70 000 gmol⁻¹, meaning ~40 nm for the corresponding alpha-helix rod. In contrast, in PBLG₃₅-PEG₅ nanoparticles, the PBLG segment corresponded to a half of this length, because the molecular weight was only 35 000 gmol⁻¹, corresponding to ~25 nm, and the nanoparticles presented an aspect ratio of 1.6, only. This tendency was also observed in PBLG₇₀-PEG₁₀-PBLG₇₀ (aspect ratio of 2.3) and PBLG₃₅-PEG₁₀-PBLG₃₅ (aspect ratio of 1.3) nanoparticles, even if the PEG segment was attached to two PBLG segments.

Interestingly, PEG₅-PBLG₇₀-PEG₅ nanoparticles showed a really elongated shape, similar to a 'rice grain', in comparation with the ellipsoidal shape of PBLG₃₅-PEG₅ nanoparticles. This could be related to the position and the quantity of PEG attached. The PBLG₃₅-PEG₅ system, was composed of 207 α -helix chains with equal amounts of PEG chains. In contrast, PEG₅-PBLG₇₀-PEG₅ system was composed of 162 α -helix chains, resulting in 323 PEG chains. Although, between both systems the number of PBLG segment was not highly different, the auto-assembly could be more 'organized' in PEG₅-PBLG₇₀-PEG₅ nanoparticles. Since the two PEG segments attached to the extremities of PBLG segment in PEG₅-PBLG₇₀-PEG₅ polymer, gives rise to a compact stacking of the chains along the long axis. Although, PBLG₇₀-PEG₁₀-PBLG₇₀ system were made of two PBLG blocks with equal PBLG segments, in comparison to PEG₅-PBLG₇₀-PEG₅ polymer, a limitation in terms of movement was observed as the nanoparticle adopted an ellipsoidal morphology, due to PBLG blocks staking along the long axis. The first copolymer induced a "mushroom type" conformation of PEG

Université Paris-Saclay Université Paris Sud/ Institut Galien Paris Sud (CNRS UMR 8612), 5 rue Jean-Baptiste Clément, 92290 Châtenay-Malabry, France chains at the surface of the nanoparticles, compared to the former triblock copolymer for which PEG chains adopted a "brush-like" conformation.

The characteristics of PBLG-based nanoparticles could be modulated by the position of PEG blocks and the molar mass of PBLG segment. In addition, the effect of additional poloxamer on PBLG-based nanoparticles not merely stabilized the surface to avoid aggregation, but had also a tendency to give rise to smaller nanoparticles.

Summarizing, the five PBLG derivatives proposed in this work could be used to prepared stable nanoparticles, whether prepared in presence or absence of poloxamer, and having distinctive architectures, both in shape and in molecular surface characteristics. Thus, these systems could interact differently within the cells in their microenvironment as well as having differentiated biophysical behavior in the blood compartment.

Optimization of Cy3-dye label concentration in PBLG-based nanoparticles

Fluorescent-labeled PBLG nanoparticles were prepared for *in-vitro* analysis purposes. In the first approach, 20% of PBLG₃₅-Cy3 was used. It was observed an aggregation tendency in all systems despite the presence of poloxamer. Thus, an optimization of PBLG₃₅-Cy3 quantity had to be performed.

The polarity of Cy3 has been reported as a factor for the self-aggregation of cyanine dyes in solution or at the solid-liquid interface. Concentration and temperature have been reported as the two main factors that enhance the aggregation of cyanine dyes (Mishra *et al.*, 2000). The experimental results in the formulation of PBLG-based nanoparticles with PBLG₃₅-Cy3 exhibited the impact of those parameters, especially in the nanoparticles based on PBLG₃₅-

Bz copolymer. It was noticed that the introduction of a quantity superior to 5% of PBLG₃₅-Université Paris-Saclay Université Paris Sud/ Institut Galien Paris Sud (CNRS UMR 8612), 5 rue Jean-Baptiste Clément, 92290 Châtenay-Malabry, France 0

Cy3, increase the size of the nanoparticles obtained. The PBLG₃₅-Cy3 percentage in the system PBLG₃₅-Bz/PBLG₃₅-Cy3 had a considerable impact on the size. System that presents a size of 239 nm, when a quantity superior to 5% of PBLG₃₅-Cy3 is used. By contrast, when the concentration was lower than 5% of PBLG₃₅-Cy3, the size dropped to 82 nm (figure 1). Therefore, to keep an appropriated level of fluorescence as well as the colloidal system stability, the amount of PBLG₃₅-Cy3 was fixed to 6%.



Figure 1. TEM micrographs at 120 kV obtained from Cy3-labeled PBLG-based nanoparticles. (a) PBLG₃₅-Bz/PBLG₃₅-Cy3 >1mg, (b) PBLG₃₅-Bz/PBLG₃₅-Cy3 <1mg.

It is important to highlight that once the quantity of PBLG₃₅-Cy3 was set, the resultant fluorescent-labeled nanoparticles with the PBLG derivatives proposed in this work conserved their 'natural' morphology, despite that an increment in terms of size is noticed.

PBLG based nanoparticles in the vascular compartment

Complement activation induced by PBLG nanoparticles

It is well established in the literature that one of the major barriers to the application of nanoparticles, is the fast clearance from the blood (and the body) by different pathways implying the immunology system. The nanoparticle recognition by the complement system can reduce the persistence of the nanoparticles in the blood and therefore decrease their efficacy (Moghimi et Szebeni, 2003; Moghimi *et al.*, 2011). It is well known that depending the morphology, including the size and the characteristics of the exposed surface, could either enhance or reduce recognition, with subsequent impact on their usefulness for pharmaceutical applications as well as their nanotoxicity (Coty *et al.*, 2017). The activation of the complement system was evaluated by C3 complement protein activation, using 2D immunoelectrophoresis, method described elsewhere (Coty *et al.*, 2016).

Within the results obtained, it was possible to observe that PBLG₇₀-PEG₁₀-PBLG₇₀ and PEG₅-PBLG₇₀-PEG₅ nanoparticles, activated slightly more the C3 protein pathway in comparison to the other systems proposed in this work. Interestingly, the shape of those particles is elongated and present the PEG segment in the extremities. However, PEG₅-PBLG₇₀-PEG₅ nanoparticles displayed a brush-type conformation and PBLG₇₀-PEG₁₀-PBLG₇₀ nanoparticles displayed a mushroom-type conformation. It can be inferred that the hydrophobic area presented in the central part of the nanoparticles could allow a slight interaction of C3 protein, due to the surface hydrophobicity at this level.

Interestingly it was also found that PBLG₃₅PEG₁₀PBLG₃₅ nanoparticles activated only moderately the complement system. Coty and colleagues (Coty *et al.*, 2017) evaluated the

activation of C3 protein in similar conditions as our analysis. Moreover, their dextran-coated poly(isobutylcyanocrylate) (PIBCA) nanoparticles presented similar architecture to PBLG₃₅PEG₁₀PBLG₃₅ nanoparticles in terms of organization of the dextran hydrophilic chains. Meanwhile, they reported an activation of C3 protein above 80% in dextran-coated PIBCA nanoparticles, while our PBLG₃₅PEG₁₀PBLG₃₅ system, barely activate the C3 protein pathway. Thus, it can be inferred that PEG layer had a better stealth profile over dextran layer, despite its conformation in the surface.

The results obtained for all systems, prepared in presence or absence of poloxamer, exhibited a low level of protein C3 activation whatever the copolymer composition at the tested concentration, highlighting the ability of these nanoparticles to be considered as candidates for drug delivery applications.

Hemolysis induced by PBLG nanoparticles

The hemolysis activity (HA) of PBLG-based nanoparticles was determined after being incubated 30 minutes in the blood. Indeed, the erythrocytes are the first element to be exposed to the nanoparticles, following their intravenous delivery. (Manaargadoo-Catin *et al.*, 2016). Nanoparticles with an aspect ratio higher than 1.3, consistently exhibited a hemolysis activity (HA) under 5%, even up to 19 nM, which level is within the requirements of the hemolytic test for biomaterials (Rao and Sharma, 1997). It can be compared to the biocompatibility determined by hemolysis activity of different drug delivery systems among the literature (Morey *et al.*, 2004; Li *et al.*, 2009; Múzquiz-Ramos *et al.*, 2013). In contrast, those with an aspect ratio less than 1.3 exhibited a higher hemolytic activity, particularly in the system based on PBLG₃₅-PEG₁₀-PBLG₃₅ with an HA₅₀ of 11.8nM.



Consistently higher hemolytic activity levels were observed for all systems after 1h of incubation, which could be attributed to the longer exposition time with a fixed quantity of erythrocytes. Even though the percentage observed overpass the recommended standard of 5% among concentrations higher than 2nM, it should be considered that once the nanoparticles are injected via intravenous pathway, a dilution process will occur spontaneously. The same concentration-exposition time dependency has been reported by Jing Zhang and colleagues in spherical oleic acid-grafted chitosan nanoparticles (Zhang *et al.*, 2012).

MTT cytotoxicity of PBLG-based nanoparticles. Comparison of the results when data are expressed in molarities or in massic concentrations (μ g/mL)

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is an extended method used to determine the *in-vitro* cytotoxicity (Burton, 2005; Van Meerloo *et al.*, 2011; Tolosa *et al.*, 2015). MTT assay is based on the conversion of MTT into formazan crystals by mitochondrial activity in metabolically active cells (Van Meerloo *et al.*, 2011). According to the literature, the appropriate selection of the cells, the sample preparation and the use of the right dose are crucial considerations for assessment the cytotoxicity level of nanoparticles by means of *in-vitro* assays (Kong *et al.*, 2011).

The MTT assay has been performed by exposing the different cell lines to suspensions of the different nanoparticle's types with increasing concentrations; at the beginning, massic concentrations were used and expressed in μ g/mL. The determination of the massic polymer concentration for each system was performed after a lyophilization step. After a comparison of the characteristics of each system evaluated, substantial differences in size and morphologies were found that could lead to a systematic error.



The studied nanoparticles are quite stable in the time of the MTT test. Thus, when exposed to nanoparticles, the possible cytotoxic events are very likely to be due to the interaction or the capture of isolated nanoparticles by the cells. In comparison, when cells are exposed to water soluble molecules, the cytotoxicity can be attributed either to molecular interactions at the membrane surface and/or diffusion within the cells cytoplasm. Therefore, we decided to compare the cytotoxicity data issued from the MTT test by expressing the concentrations of nanoparticles, either in massic concentrations or in nanoparticles "molarities" For this approach, the analysis performed in chapter 2 become an excellent tool to adequate the selection of concentrations to be evaluated.

Figure 2, shows the results of MTT assay of PBLG-based nanoparticles performed on RAW 264.7 cell line at 48h expressed either in massic concentrations in µg/mL (Figure 2-A) or in nanoparticles "molarities" (figure 2-B). As explained before, the aim for expressing the concentration in molarity terms was driven by the fact that due to size and shape heterogeneities between the different types of nanoparticles, it was necessarily to normalize the data in term of numbers (or "molarities") of nanoparticles. To illustrate this approach, it is interesting to remark that the system PBLG₃₅-PEG₅ with a size by DLS of 42 nm will be present an equivalent number of particles (i.e. in nanoparticles molarity) to a system with higher size as the case of PBLG₃₅-PEG₁₀-PBLG₃₅ with a size by DLS of 87 nm, but with an elongated shape. For determining the molarity, the nanoparticle volume calculated form dimensional TEM data, PBLG density and Avogadro number, were considered for the calculation.



Figure 2. Comparison of MTT expression in A) μ g/mL and B) molarity of PBLG-based nanoparticles.

In figure 2-A, it was possible to observe that translating the massic concentrations into μ g/mL are not equal for each system. As an example, the concentration in μ g/mL for the molarity of 2.0 E-09 in the system PBLG₃₅-Bz is 29.4 μ g/mL, in contrast, the same molarity in the system PBLG₃₅-PEG₅ is equals to 8.35 μ g/mL. This substantial difference is related with the quantity of polymer chains conforming each nanoparticle. For PBLG₃₅-Bz, it is found that each nanoparticle is formed by 514 α -helix and for PBLG₃₅-PEG₅ by 285 α -helix.

Taking in account that is in fact the individual nanoparticle that is contact with the cell, evaluated MTT assay in terms of concentration can lead a systematic difference in the number of nanoparticles used. Therefore, MTT assay in the present work was valuated in terms of Molarity based in number of nanoparticles per system.

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Cytotoxicity and cellular uptake of PBLG nanoparticles

The results obtained after MTT determination, showed that HUVEC and RAW 264.7 cells remained with a favorable cell viability beyond 80% at 24h and 48h after exposition to PBLG₃₅-Bz system, regardless the presence or absence of poloxamer. Hence, PBLG-based nanoparticles would avoid non-specific accumulation in endothelium cells or recognition by the immune system and, therefore they will persist in circulation.

Interestingly, PBLG-based nanoparticles could interact with prostate cancer cells (PC3) and to a less extent with melanoma cells (B16); Indeed, a higher cytotoxicity was evidenced after nanoparticle exposition. As an example, PEG₅-PBLG₇₀-PEG₅ system, with an aspect ratio of 2.6, presented an IC₅₀ of 4nM in PC3, in comparison with the projected IC₅₀ of 25nM in B16.

It was found that spherical nanoparticles, meaning aspect ratios ranging from 1.1 to 1.3 were more cytotoxic in both cancer cell lines investigated (PC3 and B16). As explained by Toy and colleagues (Toy *et al.*, 2013), the spherical morphology and the size of those systems contributes to enhance cell interaction. However, the presence of PEG chains modifies this behaviour. The system PBLG₃₅-Bz despite the presence of poloxamer was highly recognized by Raw 264.7, which are a model of macrophages, with an IC₅₀ of 14nM. In comparison with PBLG₃₅-PEG₁₀-PBLG₃₅ system, the high density of PEG chains may avoid any cell recognition even if both systems have an aspect ratio below 1.4.

The understanding of the impact of the architecture and the shape on cellular/nanoparticle interactions has been undertaken. The analysis was carried out on Cy3-labeled PBLG nanoparticles prepared in presence and absence of poloxamer. It is important

to remember that the role of poloxamer is to hydrophilize the whole surface of the nanoparticles, making possible to investigate separately the effect of the surface parameter from the size and shape parameter.

Positively, it was found that PBLG based nanoparticles, regardless their architecture or the presence/absence of poloxamer in surface, interacted poorly with Huvec endothelial cells model. This result suggested that PBLG-based nanoparticles could avoid unspecific accumulation in these cells while remaining available for interacting with the selected targeted cells during their complete circulation life-time.

For the different cell types which were investigated, it was noticed that the intensity of the capture was considerably influenced by the aspect ratio and secondly by the PEG molecular conformation at the surface (brush versus loops) both without and when poloxamer was added. Thus, the nanoparticles with a spherical morphology and those with a "mushroom-like" PEG conformation at the surface were more recognized. However, in the absence of poloxamer, the interaction resulted from the conjunction of aspect ratio and surfaces characteristics. According to the literature, this tendency could be the result of the presence of hydrophobic patches at the surface of the nanoparticles in absence of poloxamer hydrophilic, thus resulting in the formation of a corona made of adsorbed serum proteins at nanoparticles surface (Alexandridis et Alan Hatton, 1995; Santander-Ortega *et al.*, 2006; Yan *et al.*, 2010). Considering the results obtained in the second set of particles, PBLG-based nanoparticles presenting simultaneously surface and shape variety, it appeared that the cellular uptake was also influenced by the aspect ratio, but it could be modulated by the balance between hydrophobic/hydrophilic surfaces.



In this work a variety of PBLG amphiphilic copolymers in di- and tri-block conformation were successfully synthetized by ring-opening polymerization. The synthetized copolymers were made of PBLG and PEG blocs with varying lengths, that were calculated based in the molar mass obtained and the intrinsic chain sizes. The PBLG chains adopted an α helix conformation adopting a rod shape with a hydrophobic nature. They were conjugated to hydrophilic PEG chains to obtain amphiphilic copolymers which were self-assembled by nanoprecipitation method to produce non-spherical nanoparticles with brush-type and mushroom-type surface PEG conformation. PBLG derivatives were successfully synthesized by ring-opening polymerization of the corresponding BLG-NCA, highlighting the possibility to access to a wide variety of copolymers just by changing the molecular initiator and controlling the synthesis conditions. One of the finding was that the distribution of the hydrophobic and hydrophilic chains in the copolymers and the functional groups presented, could modulate the architecture of the nanoparticles obtained. This allowed us to design new polymeric derivatives guiding to a desired architecture.

Two set of PBLG-based nanoparticles were prepared, one in presence of poloxamer and the second in absence of poloxamer, a triblock copolymer that can be strongly absorbed in hydrophobic surfaces. Stable and reproducible nanoparticles prepared in presence of poloxamer were successfully obtained with sizes under 100 nm and negative ζ potential. In addition, PBLG-based nanoparticles prepared in absence of poloxamer exhibit sizes under 100 nm with higher negative ζ potential, corresponding to the surface anisotropy in the absence of any added poloxamer. The TEM analysis revealed a variety of morphologies ranging from almost spherical particles (aspect ratio 1.3) to ellipsoidal shape (aspect ratio 2.6). It was also found that the morphology was preserved wherever poloxamer was used or not during the preparation process. Through surface analysis, it was determined that PBLG-based nanoparticles exhibited an amphiphilic nature, particularly in the case of elongated tri-block nanoparticles.

Interestingly, these unusual characteristics could be modulated through modifications of the copolymer conformations, which in turn impacted the shape and surface characteristics of the nanoparticles. Not surprisingly, the interactions of the nanoparticles with cells belonging to different cells types encountered in the vascular compartment, including endothelial cells, erythrocytes, macrophages, PC3 and B16 cells (prostate tumoral cells and melanoma). Low cytotoxicity, very moderate hemolysis activity as well as limited complement activation levels, composed a set of encouraging results. Meanwhile, in nanoparticles presenting surface/shape variations, the cellular uptake varied accordingly to the aspect ratio, i.e. the geometry of the particles, as well as the hydrophobic/hydrophilic balance of their surface. Generally speaking, low endothelial capture was found which was favorable since the lowest unspecific interactions toward normal endothelium is requested not merely to reduce toxicity but also to have longer circulation life-time once administrated. In addition, PBLG₃₅-PEG₅ and PEG₅-PBLG₇₀-PEG₅ systems presented the best profiles among the systems evaluated in this work. Finally, attempts were made to gain specific recognition of B16 cells by grafting the MART-1 recognition ligand at the nanoparticles surface. Although specific recognition of B16 cells occurred, the density of MART-1 ligand should still be optimized before undertaking in vitro and in vivo testing of the capacity of these particles to recognize and intercept specific cells within the blood stream.

In summary, PBLG-based nanoparticles present attractive characteristics for nanomedicine applications due to the specific characteristics of the rigid and hydrophobic



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GENERAL CONCLUSIONS AND

PERSPECTIVES

GENERAL CONCLUSION AND PERSPECTIVES

It is well-known that cancer is one of the leading causes of morbidity and mortality worldwide and the tendency of new cases each year is in augmentation. Several strategies have been developed in order to treat cancer, among them is to target metastasis. The spread of cancer cells from the primary tumor can be released at the first stage of tumor growth and is causing 90% of cancer-related deaths worldwide. The tumor spread is achieved by the release of circulating tumor cells in a multidirectional process. Regarding that only 0.01% of CTCs shed will form successful secondary tumors and those are 10 to 10 billion of normal cells approximately, the challenge is immense. However, in the recent years, cell/particle's margination is gaining attraction to be considered as a key factor to target CTCs. The margination of particles can be influenced by several factors such as size, shape, volume, density, etc. Thus, the nanoparticle's design is currently taking more in consideration the architecture's effect in margination to target CTCs and how the coupling of several molecules that are related with the metastasis could boost their activity.

Following the approach of nanoparticles rational design, the main objective of this work is to develop polymeric nanoparticles with modulated architecture to be proposed as a strategy to target CTCs. For this objective, it was successfully synthetized a variety of PBLG derivates in di- and tri-block conformation by ring-opening polymerization. The synthetized copolymers exhibit a range of PBLG and PEG lengths, that were calculated based in the molar mass obtained and the intrinsic chain sizes. The α -helix conformation of PBLG chain with a hydrophobic nature, combined with hydrophilic PEG chain allowed to obtain self-assemble amphiphilic nanoparticles with sizes under 100 nm and negative ζ potential, from rod (aspect ratio 1.3) to ellipsoidal shape (aspect ratio 2.6) and hairy-type and mushroom-type surface PEG conformation by nanoprecipitation method. Considering that each nanoparticle was formed by PBLG and PEG polymers, it is interesting to remark that the distribution of the hydrophobic and hydrophilic chain parts in the polymer structure and the functional groups presented, could modulate the architecture obtained.

Based in our results, the tendency in cellular uptake of PBLG-based nanoparticles with similar surface is ordered mainly by the aspect ratio. Meanwhile, in nanoparticles presenting a surface/shape variety, the cellular uptake also followed a tendency ordered by the aspect ratio, but it could be modulated through a hydrophobic/hydrophilic balance of the proposed surface. Furthermore, low endothelial uptake ratio, low activation of complement system and low hemolysis activity were found as a positive indication of their well biocompatible profile and their low non-specific interaction toward normal tissue, allowing PBLG-based nanoparticles not merely to reduce secondary effects but also to have longer circulation life-time once administrated.

Regarding the systems proposed in this work, PBLG₃₅-PEG₅ and PEG₅-PBLG₇₀-PEG₅ systems presented the best profiles among the systems evaluated in this work. Despite that all systems have low endothelial uptake ratio, low activation of complement system and low hemolysis activity, PBLG₃₅-PEG₅ and PEG₅-PBLG₇₀-PEG₅ systems also present low macrophage uptake, when prepared in absence of poloxamer. In detail, PEG₅-PBLG₇₀-PEG₅ system present a trend towards PC-3>B16>Raw 264.7>HUVECs. Although the uptake ratio is considering low, this behavior could be modified trough antibody coupling on the PEG chains. In the case of PBLG₃₅-PEG₅, the trend is towards PC-3>B16>Raw 264.7>HUVECs. Those systems prepared in aqueous suspension presents a surface/shape variety more interesting than the same systems prepared in presence of poloxamer.

In summary, PBLG-based nanoparticles present attractive characteristics to nanomedicine applications. Their potential use, is not merely based on their well-defined biocompatible profile or their stability through time, it is their potential capacity to reorganize and modulate their architecture and fate in the body that make them solid candidates for rational-designed nanomedicines to target CTCs.

RESUME ETENDU

INTRODUCTION

En dépit de progrès considérables, le cancer constitue une des principales causes de morbidité et de mortalité dans le monde. Le traitement des cancers métastatiques représente un énorme défi puisqu'on estime actuellement que 90% des décès liés au cancer sont causés par la propagation de cellules cancéreuses d'une tumeur primitive vers des organes distants. Une fois implantées et disséminées dans des organs distants, les métastases sont beaucoup plus difficiles à éliminer par les moyens tels que l'ablation chirugicale, la destruction des cellules tumorales par irradiation et bien sur les traitements medicaments, chimiothérapeutiques et/ou immunologiques.

Actuellement, on s'accorde à estimer que l'envahissement d'organes distants de la tumeur primitive par des cellules cancéreuses a pour origine l'échappement continu et régulier de certaines cellules tumorales provenant des tumeurs primitives. Ces cellules empruntent les systèmes circulatoires, lymphatiques et sanguins, ce qui contribue à leur distribution dans l'ensemble de l'organisme et finalement à leur dissémination dans certains organes distants dans lesquelles certaines d'entre elles parviennent à s'infiltrer. Pour cette raison, les cellules tumorales circulantes (CTCs) sont considérées comme étant des biomarqueurs utiles au diagnostic précoce de certains cancers ou bien de leur récidive, à l'évaluation de l'efficacité des traitements, à la personnalisation des traitements, etc. Bien qu'au plan pratique la detection des CTCs soit techniquement très delicate, notamment en raison de leur grande rareté en comparaison avec les éléments figures du sang, différentes techniques ont été mises au point ces dernières années et sont actuellement disponibles.

Dans ce contexte, nous avons voulu mettre à profit les connaissances relatives aux CTCs, non plus uniquement dans un but diagnostic, mais dans le but de bloquer leur propagation vers les organes distants. Ainsi, dans le cadre de ce travail doctoral, nous avons imaginé des systemes capables d'intercepter de manière sélective les cellules tumorales circulantes lorsqu'elles se trouvent dans l'environnement circulatoire. Nous avons émis l'hypothèse que lorsque les CTCs se trouvent transitoirement dans le sang, elles deviennent plus facilement accessibles aux agents thérapeutiques, et donc plus vulnérables qu'elles ne le sont au cours de leur migration au sein des organes. Afin de verifier cette hypothèse, nous avons sélectionné des nanoparticules polymères à architecture contrôlée et fonctionnalisables. En effet, les nanoparticules polymères présentent une série de caractéristiques intéressantes puisque: (i) la surface et la morphologie ces objets peuvent être ajustées de manière à assurer leur circulation prolongée dans le compartiment sanguin, (ii) une fonctionnalisation adequate de leur surface peut leur conférer la capacité de reconnaitre spécifiquement certaines cellules, (iii) elles ont la capacité de véhiculer des entités cytotoxiques qui leur sont associées, puis de les relarguer de manière contrôlée. Dans ce cadre, notre travail doctoral a consisté à fabriquer et caractériser des nanoparticules possédant les propriétés recherchées. Dans un deuxième temps, ces nanoparticules ont été fonctionnalisées par la protéine MART-1 reconnue par les cellules B16, une lignée cellulaire classiquement utilisée en tant que modèle du mélanome. Leur efficacité après injection intraveineuse devra toutefois être précisée in vivo.

1. Les cellules tumorales circulantes et leur ciblage au moyen de nanomédecines en vue de prévenir l'apparition des métastases

Comme expliqué ci-dessus, la propagation de cellules cancéreuses provenant d'une tumeur primaire dans des organes distants est un mécanisme essentiel dans le processus métastatique. Ainsi, il est maintenant établi pour certains cancers que la détection des cellules tumorales circulantes chez les patients est liée à un pronostic sombre et à de faibles taux de survie. Nous avons donc proposé dans un premier temps une revue des évènements concernant la migration des CTCs dans le sang, en nous focalisant sur l'étape cruciale de la margination cellulaire lors de la circulation des CTCs dans le sang. Dans un second temps, nous avons passé en revue les quelques systèmes conçus pour interagir avec les CTCs et décrits dans la littérature.

2. Nanoparticules de poly(y-benzyl-L-glutamate) à architecture contrôlée

Les développements actuels des nanomédecines et en particulier des nanoparticules polymères ont été facilités par une conception plus rationnelle des particules. Il apparaît de mieux en mieux que la morphologie des objets (taille, forme géométrique, caractéristiques de surface) ainsi que leur architecture interne, gouvernent profondément leur distribution dans l'organisme. Pour cette raison, nous avons opté pour la construction de nanoparticules multi-fonctionnalisées grâce à une technique d'auto-assemblage de copolymères amphiphiles préalablement synthétisés. Les copolymères sélectionnés sont des copolymères amphiphiles di- et tri-blocs, comprenant un ou deux blocs hydrophobes constitués de poly(glutamate de benzyle), tandis que le bloc hydrophile était constitué d'un bloc hydrophile de poly(éthylène glycol). Le bloc poly(glutamate de benzyle) a été synthétisé avec succès par ouverture de cycle de la lactone correspondante. Les copolymères synthétisés présentaient une gamme de longueurs de PBLG et de PEG. La conformation α -hélice de la chaîne PBLG de nature hydrophobe, associée à la chaîne PEG hydrophile confère à ces copolymers un caractère amphiphile qui autorise leur auto-assemblage par nanoprecipitation. Il est ainsi possible de preparer des nanoparticules amphiphiles de taille inférieure à 100 nm et de potentiel ζ négatif,



dont la géométrie peut être contrôlée, allant de la forme sphérique (rapport d'aspect 1.3) à la forme ellipsoïdale (oblats) (rapport d'aspect 2,6) et qui présentent en surface des chaînes de PEG sous des conformations et des densités de surface contrôlées. On estime ainsi que la conformation des chaînes de PEG se localisent en surface des particules et adoptent soit un type « poilu » lorsque les chaînes PEG sont libres, soit un type « champignon » lorsque des copolymères PBLG-PEG-PBLG sont utilisés. Ces nanoparticules sont stables et reproductibles.

Deux séries de nanoparticules à base de PBLG ont été préparées, l'une en présence de poloxamer et la seconde en absence de poloxamer, un copolymère tri-bloc qui peut être fortement absorbé dans les surfaces hydrophobes. De la même façon que précédemment, des nanoparticules stables et reproductibles ont été préparées avec succès en présence de poloxamer avec des tailles inférieures à 100 nm et un potentiel ζ négatif. Ainsi, des nanoparticules à base de PBLG préparées en l'absence de poloxamer, présentent des tailles inférieures à 100 nm avec un potentiel ζ négatif plus élevé. L'augmentation du potentiel ζ négatif est le reflet de l'absence d'absorption de poloxamer dans la surface hydrophobe exposée. L'analyse TEM a révélé une variété de morphologies de sphérique (rapport d'aspect 1,3) à ellipsoïdale (rapport d'aspect 2,6). Il a également été constaté que la morphologie est préservée malgré l'utilisation du poloxamer dans le processus de préparation. Grâce à l'analyse de surface, il a été déterminé que les nanoparticules à base de PBLG présentent un caractère amphiphile, en particulier dans les nanoparticules tri-blocs ellipsoïdales.



3. Interactions des nanoparticules de poly(γ-benzyl-L-glutamate) avec différents types cellulaires représentatifs du compartiment sanguin. Impact de leurs caractéristiques morphologiques

Les nanoparticules préparées précédemment ont été conçues pour circuler dans le compartiment sanguin, avec l'objectif d'intercepter efficacement les CTCs. En effet, étant donné la nature liquide du serum, les nanoparticules devraient avoir une mobilité accrue comparativement à des organes denses. Pour cette raison, la probabilité d'interaction avec les mesurer les capacités d'interaction de ces mêmes nanoparticules avec les différents éléments figures du sang, ainsi que les endothéliums vasculaires, puisque celles-ci doivent être minimisées afin d'augmenter leur spécificité d'action vis à vis des cellules ciblées.

L'impact de la modification de l'architecture nanoparticulaire sur les capacités d'interactions des différentes nanoparticules préparées, d'une part avec les protéines plasmatiques et, d'autre part, avec les différents types cellulaires rencontrés dans le compartiment sanguin a donc été évalué. Pour cela, deux séries de nanoparticules à base de PBLG et marquées à la cyanine 3 (Cy3) ont été préparées. La première série était constituée de nanoparticules préparées directement dans l'eau, tandis que la deuxième série a été préparée dans une solution diluée d'un copolymère amphiphile de type PEG-PPO-PEG (poloxamer). Ainsi, la surface des nanoparticules appartenant à cette deuxième catégorie sont uniformément recouvert des chaînes de PEG, tandis que les nanoparticules de la première série présentent une surface anisotrope en terme d'hydrophilie/lipophilie.

Des études d'interactions ont été menées sur différentes cultures cellulaires. Les résultats les plus marquants montrent que l'élongation des nanoparticules (oblats) et

l'anisotropie de leur surface, caractérisée par leur balance hydrophile/lipophile, gouvernent profondément leurs interactions. De manière fort intéressante, il apparaît que l'élongation des particules dont la surface est uniformément hydrophile diminue l'intensité de leur capture par les différents types cellulaires modèles étudiés (HUVECs modèle de cellules endothéliales), cellules RAW 276.7 (modèle de macrophages) et cellules PC3 (cancer de la prostate) et B16 (mélanome). En revanche, lorsque ces nanoparticules présentent une anisotropie de surface, leur capture par ces différents types cellulaires est augmentée avec l'élongation des particules (facteur d'élongation de 2,1).

De plus, ces nanoparticules présentaient une faible cytotoxicité, une activité d'hémolytique modérée vis-à-vis des hématies. Par ailleurs, la capacité des différents types de nanoparticules d'activer le système du complément s'est révélée peu élevée.

4. Préparation d'immuno nanoparticules conçues pour l'interception des CTCs dans le flux sanguin

Dans une seconde étape, les nanoparticules préparées ont été fonctionnalisées avec l'anticorps MART-1, capables de reconnaître les cellules B16, un modèle de mélanome largement utilisé. Des immunonanoparticules conjuguées avec l'anticorps MART-1 ont été préparées en deux étapes. Dans un premier temps des nanoparticules constituées de copolymères du poly (γ-benzyl-L-glutamate) (PBLG) et de PEG-biotine ont d'abord été préparées par la méthode de nanoprécipitation, puis dans un deuxième temps, ces nanoparticules ont été conjuguées à l'anticorps MART-1 au moyen d'un couplage de type PEGbiotine-streptavidine-biotine-MART-1. La conjugaison de cet anticorps sur la surface des nanoparticules a été évaluée par Western blot. Les caractéristiques morphologiques des nanoparticules ont été évaluées in vitro. Ensuite, leurs capacités d'interaction avec les cellules de mélanome B16-GFP d'une part et avec les cellules HUVECs (modèle de cellules endothéliales issues de la veine ombilicale humaine) d'autre part ont été mesurées. L'activation du complément a été étudiée par immunoélectrophorèse bidimensionnelle. Les nanoparticules présentaient des tailles comprises entre 20 et 100 nm et une charge de surface négative (-3 à -30 mV). La conjugaison de l'anticorps sur les surfaces des nanoparticules a été détectée par la technique de transfert de Western blot et confirmée par les changements dans la taille des particules et la charge de surface. Les nanoparticules développées n'étaient pas capables d'activer le système du complément, ce qui suggère que ces nanoparticules pourraient posséder des temps de circulation importants in vivo. En ce qui concerne l'analyse in vitro, les particules présentaient une faible cytotoxicité lorsqu'elles étaient testées dans des cellules B16-GFP et HUVEC. Dans les essais de capture cellulaire, les immuno-nanoparticules, présentant l'anticorps spécifique MART-1 pour la reconnaissance de l'antigène surexprimé dans les cellules B16 de mélanome, ont montré une augmentation de 40 à 50% de l'interaction avec ces cellules, indiquant une certaine spécificité de ces nanoparticules. Toutefois, ces premiers résultats devraient être complétés par une recherche systématique de la quantité d'anticorps optimale à coupler, avant d'envisager d'utiliser ces particules dans un modèle in vivo.

Conclusion générale et perspectives

Le présent travail représente une des toutes premières tentatives consistant à concevoir des nanoparticules conçues pour intercepter les CTCs directement dans le compartiment sanguin. Les copolymères amphiphiles basés sur l'emploi de blocs PBLG ont démontré leur capacité de moduler l'architecture des nanoparticules, permettant notamment d'accéder à des morphologies non sphériques. En effet, l'élongation des particules semble être favorable à la

margination des nanoparticules, c'est à dire leur concentration dans la vicinité de l'endothélium vasculaire. Cette situation est favorable au ciblage des CTCs lorsque celles-ci entreprennent leur extravasation, avant de rejoindre les organes distants.

En ce qui concerne les systèmes proposés dans ce travail, les systèmes PBLG₃₅-PEG₅ et PEG₅-PBLG₇₀-PEG₅ ont présenté les meilleurs profils parmi les systèmes évalués. Bien que les différents types de nanoparticules présentent tous un faible taux d'interaction endothéliale, une faible activation du système du complément et une faible activité hémolytique, les systèmes PBLG₃₅-PEG₅ et PEG₅-PBLG₇₀-PEG₅ présentaient également une faible interaction avec des macrophages lorsqu'ils sont préparés en absence de poloxamer. En revanche les nanoparticules constituées de PEG₅-PBLG₇₀-PEG₅ étaient considérablement mieux capturées par les cellules PC-3 (modèles de cancer de la prostate) et B16 (modèle de mélanome)

En résumé, les nanoparticules à base de PBLG présentent des caractéristiques intéressantes pour les applications en nanomédecine. Les nanoparticules constituées de copolymères du PBLG se prêtent également facilement à leur immuno-conjugaison. Toutefois, celle-ci devrait être optimisée avant de pouvoir envisager la réalisation d'essais d'interceptions de CTCs dans des modèles in vivo.



CURRICULUM VITAE

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R&D FORMULATION SCIENTIST DRUG DELIVERY AND NANOMEDICINE APPLICATIONS

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	Language skills:		WORK EXPERIENCE & PROJECTS
\checkmark	English (Fluent)	April 2016-	Nanomedicine Junior Experimental Project Manager
\checkmark	French (Fluent)	March 2017	Université Paris-SUD Saclay
\checkmark	Portuguese (Fluent)	Paris,	2
✓	Spanish (Native)	France	 Project: Evaluation of nanoparticle architecture for circulating tumor cells in metastasis treatment.
	<u>Techniques expertise:</u>		✓ Project : Stability and biocompatibility evaluation of PBLG-based
✓	Size Exclusion Chromatography		nanoparticles in <i>in-vitro</i> blood conditions.
✓.	Nuclear Magnetic Resonance		
✓	Infrared	Keywords:	Formulation, in vitro assays, biocompatibility, nanoparticles, poly-peptide,
✓.	Dynamic Light Scattering		experimental design, cytotoxicity, oncology, technology transfer, proof-of-
✓	Transmission Electronic		concept, technical leadership, mentoring, project management, pro-active,
,	Microscopy		materials science, innovation, creativity, adaptation capacities, consulting.
✓	Isothermal Titration Calorimetry		
✓.	Polymer-peptide synthesis	November	Nanomedicine Junior Experimental Project Manager
✓	In-vitro assays	2014-	Université Paris-SUD Saclay
✓	Flux Cytometry	March	
✓	Biocompatibility assays	2016	✓ Project: Formulation of novel polymeric nanoparticles for melanoma
~	Manipulation of biologic	Paris,	cancer treatment.
	samples (bacteria, parasite and	France	
	fungı, blood).		Formulation, antibodies conjugation, biocompatibility, cellular up-take,
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Capacity to work under-pressure Versatility and creativity Pro-active Adaptation capacities Client consulting		Monterrey, Mexico Keywords:	Formulation, commercial polymer, pH material response, sample stability, antifungal encapsulation, protocol design, interlaboratory collaboration, creativity, pro-active, versatility.		
August 2008- August 2010 Hermosillo, Mexico	 Nanotechnology and Material Science Laboratory Research Assistant Universidad de Sonora ✓ Project: Synthesis and characterization of multifunctional polymeric nanoparticles prepared from Poly(-γ-benzyl L-glutamate) derivatives. 				
Keywords:	Multifunctional nanoparticles, polymer synthesis, amphiphilic poly-peptide, drug encapsulation, formulation, experimental design, nanoparticle characterization, data interpretation, report redaction, budget and resources planning, trouble-shoot polymer production, creativity, pro-active, mentoring, interdisciplinary communication, technical leadership.				
July 2009- August 2009 La Paz, Mexico	Medical Laboratory Scientist Institute for Social Security and Services for State Workers				
Keywords:	Sample collection, biomedical analysis, hospital work experience, blood bank processing, emergency unit, data interpretation, report redaction, pro-active, interdisciplinary communication.				
August 2003- August 2005 La Paz, Mexico	Laboratory Teaching Assistant Jóse María Morelos y Pavón CCH-UNAM College				
Keywords:	Protocol design, science mentoring, interdisciplinary communication, budget management, technical leadership.				
EDUCATION 2013 - Present Paris, France 2013 - Present Hermosillo, Mexico 2010 - 2013 Hermosillo, Mexico 2005 - 2010 Hermosillo, Mexico	Doctor of Philo Université Paris Doctor of Philo Universidad de Master's Degre Universidad de Bachelor's Deg Universidad de	sophy (Ph.D), -SUD Saclay sophy (Ph.D.), Sonora ee, Materials S Sonora ree, Medical I Sonora	Therapy Innovation Materials Science, Nanomedicine cience, Nanomaterials aboratory Scientist		
Associations		- Soft Cond	ensed Matter Thematic Network-CONACyT, member since 2012.		
Training	– GPC – Tra	Training Scho	ol for Separations. Customer Training Course. Malvern Instruments. 17-18 October 2012 . Houston, United States of America. tron Microscopy full training formation, Platform IMAGERIE CNRS, 1-4 September 2015. Gif-sur-Ivette, France.		

ReferencesPr. Gilles Ponchel. Professor of Université de Paris-SUD, France.
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- Further references upon request.

Hobbies

- Reading and writing, natation, handicrafts, travel and languages.

UNIVERSITE PARIS-SACLAY



Titre : Nanoparticules à base de poly(glutamate de benzyle) pour l'interception et la destruction des cellules tumorales circulantes dans la circulation sanguine

Mots clés : copolymères amphiphiles du poly(benzyle glutamate), nanomédecine, architecture nanoparticulaire, mélanome, cellules tumorales circulantes (CTCs), Cellules PC3, Cellules B16, MART1

Résumé : En dépit de progrès considérables, le cancer reste l'une des principales causes de morbidité et de mortalité dans le monde. Actuellement, 90% des décès liés au cancer sont causés par la propagation de cellules cancéreuses vers des organes distants. Une fois implantées et disséminées, les métastases sont beaucoup plus difficiles à détruire par les moyens de la chimiothérapie.

la suite d'un processus А d'intravasation, certaines cellules tumorales s'échappent de la tumeur primaire et empruntent les systèmes circulatoires avant d'être ensuite extravasées, puis distribuées et finalement disséminées dans divers organes. Ainsi, dans l'environnement circulatoire, ces cellules tumorales circulantes (CTCs) se trouvent particulièrement accessibles aux agents thérapeutiques. Dans ce cadre, nous avons d'utiliser des nanoparticules imaginé à architecture contrôlée, afin d'intercepter de sélective cellules manière ces dans l'environnement sanguin.

Dans cet objectif, nous avons synthétisé par ouverture de cycle de la lactone correspondante des copolymères amphiphiles diet tri-blocs du poly(glutamate de benzyle). Leur auto-assemblage a permis d'obtenir des nanoparticules amphiphiles de taille inférieure à 100 nm et de potentiel ζ négatif, dont la géométrie contrôlable va de la forme sphérique (rapport d'aspect 1.3) à la forme ellipsoïdale (oblats) (rapport d'aspect 2,6) et qui présentant en surface des chaînes de PEG sous des conformations et des densités de surface contrôlées.

En raison de leur capacité de circuler dans le compartiment sanguin, ces nanoparticules ont une probabilité d'interaction optimale avec les CTCs.

L'impact de la modification de leur architecture a été établi en étudiant les capacités d'interactions des différentes nanoparticules préparées, d'une part avec les protéines plasmatiques et d'autre part, avec les différents types cellulaires rencontrés dans le compartiment sanguin.

Les résultats les plus marquants montrent que l'élongation des nanoparticules (oblats) et l'anisotropie de leur surface, caractérisée leur balance par hydrophile/lipophile, gouvernent profondément leurs interactions. De manière fort intéressante, il apparaît que l'élongation des particules dont la surface est uniformément hydrophile diminue l'intensité de leur capture par les différents types cellulaires modèles étudiés (HUVECs modèle de cellules endothéliales), cellules RAW 276.7 (modèle de macrophages) et cellules PC3 (cancer de la prostate) et B16 (mélanome). En revanche, lorsque ces nanoparticules présentent une anisotropie de surface, leur capture par ces différents types cellulaires est augmentée avec l'élongation des particules (facteur d'élongation de 2.1).

Dans un dernier volet expérimental, ces nanoparticules ont été modifiées par greffage de la protéine MART1 à leur surface. Ces immunonanoparticules ont montré une certaine capacité de reconnaissance des cellules B16 (modèle du mélanome). Leur efficacité après injection intraveineuse devra toutefois être précisée *in vivo*.





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Title: Poly(benzyle glutamate)-based nanoparticles for intercepting and destroying circulating tumor cells into the bloodstream

Keywords: Poly(benzyl glutamate) amphiphilic copolymers, nanomedecine, architecture of nanoparticles, melanoma, circulating tumoral cells (CTCs), MART1

Abstract: Despite the considerable progress, cancer remains one of the leading causes of morbidity and mortality worldwide. Currently, 90% of cancer deaths are caused by the spread of cancer cells to distant organs. Once implanted and disseminated, metastases are much more difficult to destroy by means of chemotherapy.

Following a process of intravasation, some tumor cells escape from the primary tumor and migrate through the circulatory systems before being extravasated, then distributed and finally disseminated in various organs. Thus, in the circulatory environment, these circulating tumor cells (CTCs) are particularly accessible to therapeutic agents. In this context, we have imagined the use of nanoparticles with controlled architecture, in order to selectively intercept these cells in the blood environment.

For this purpose, we have synthesized by ring opening of the corresponding lactone, amphiphilic di- and tri-block copolymers of poly (benzyl glutamate). Their self-assembly made it possible to obtain amphiphilic nanoparticles smaller than 100 nm in size and with a negative ζ potential, whose controllable geometry ranges from spherical (aspect ratio 1.3) to ellipsoidal (oblates) (aspect ratio 2, 6) and having PEG chains on the surface under controlled surface conformations and densities.

Due to their ability to circulate in the blood compartment, these nanoparticles have an optimal probability of interaction with CTCs. The modification impact of their architecture has been established by studying the interaction capacities of the different nanoparticles prepared. On the one hand with the plasma proteins and on the other hand, with the different cell types encountered in the blood compartment.

The most striking results show that the elongation of the nanoparticles (oblates) and the anisotropy of their surface, characterized by their hydrophilic / lipophilic balance, strongly govern their interactions. Interestingly, it appears that the elongation of particles whose surface is uniformly hydrophilic decreases the intensity of their capture by the different types of cell models studied (HUVEC model endothelial cells). RAW 276.7 cells (macrophage model) and cells PC3 (prostate cancer) and B16 (melanoma). Although, when these nanoparticles exhibit surface anisotropy, their capture by these different cell types is increased with the elongation of the particles (elongation factor of 2.1).

In a final experimental part, these nanoparticles were modified by grafting the MART1 protein on their surface. These immuno-nanoparticles showed a certain recognition capacity of B16 cells (melanoma model). However, their efficacy after intravenous injection should be specified *in vivo*.

