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pH-sensitive double-hydrophilic block copolymer micelles for biological applications

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ABSTRACT

In the recent years, double-hydrophilic block copolymer (DHBC) micelles have appeared as potential vectors for pharmaceutical applications due to their simple preparation method in aqueous solvent. The present study aims at underscoring the strategy for the choice of the partners in the formulation of DHBC micelles presenting a good stability in physiological conditions (pH 7.4, 0.15 mol/L NaCl) and a pH-sensitivity allowing their disassembly at pH 5. Using light scattering and Laser-Doppler electrophoresis, micelles of polymethacrylic acid-*b*-polyethylene oxide complexing either poly-L-lysine (PLL) or an oligochitosan were characterised. Whatever the polyamine counter-polyion considered, the micelles were perfectly formed for an amine/methacrylic acid molar charge ratio of one. They were characterised by a hydrodynamic diameter of 28 nm for PLL and 60 nm for oligochitosan and by a neutral zeta potential. The stability study as a function of the pH and of the ionic strength revealed different behaviours. Oligochitosan micelles were stable until pH 7 and unstable at 0.15 mol/L NaCl. On the contrary, PLL micelles were stable in physiological conditions and disassembled at pH 5. As a conclusion, the choice of the partners to formulate double-hydrophilic block copolymer based-micelles is strategic in order to obtain well-adapted vectors applied to the pharmaceutical field.

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1. Introduction

Micelles are composed of amphiphilic molecules organised into supramolecular assemblies. These molecules can be surfactants or amphiphilic block copolymers. The objects are constituted of an hydrophobic core, which is stabilised by the hydrophilic part of the amphiphilic system. We are particularly interested in double-hydrophilic block copolymer (DHBC) based-micelles, which are formulated between an ionic–neutral DHBC and an oppositely charged counter-polyion (Sanson et al., 2004; Baccile et al., 2008; Boudier et al., 2009a). DHBC polymers are water-soluble and they are not able to form aggregates by themselves, in standard conditions. The formation of micelles of ionic–neutral DHBC polymers can be induced by forming a coacervate due to electrostatic interactions between the polyanions or the polycations of the DHBC polymers and the counter-polyion (Cohen Stuart et al., 1998; Van Der Burgh et al., 2004; Gerardin et al., 2003). The non-charged block of the copolymer (often a classical polyethylene oxide) forms a stabilising corona surrounding the insoluble coacervate core in order to obtain polyion complex micelles or complex coacervate micelles. An interesting range of properties is nowadays attributed to those assemblies as far as the pharmaceutical field is concerned. Compared to surfactant micelles, they show a best stability in physiological fluids due to their small critical association concentrations (Kabanov and Alakhov, 1996). Furthermore, these objects are characterized by a size of a few tens of nanometers, a lot less than that of liposomes, allowing a better access to inflammatory or to tumorous sites (Nishiyama and Kataoka, 2006). Finally, the polyethylene oxide corona avoids particle opsonization by the reticuloendothelial system promoting a long lasting circulation within the organism (Gref et al., 1994; Stolnik et al., 1994; Owens and Peppas, 2006) for *in vivo* applications. The nature of the core blocks is important because it confers peculiar properties to the micelles. Indeed, polyion complex assemblies can respond to various environmental *stimuli*: temperature, ionic strength or pH (Andre et al., 2005; Solomatin et al., 2003; Cheng et al., 2008), depending on the copolymer nature. Using a weak acid or a weak base as the charged part of the copolymer or as the counter-polyion offers the possibility to disassemble the micelles (Baccile et al., 2008), for example, in an acidic environment, as in some cell compartments or in peculiar tissues such as the tumors (Nishiyama and Kataoka, 2006) and it also guarantees the stability at physiological pH. Such micelles therefore appear as powerful flexible candidates since a wide panel of copolymers and counter-polyions are at the disposal of the researchers. Then, the choice of the different micelle partners is strategic since

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it must be well-adapted to a biological application that requires properties such as: drug encapsulation, pH-sensitivity, stability, and specific size distribution. Our aim is to formulate micelles able to entrap a therapeutic drug, which can be a charged molecule or not as it does not need to act as the counter-polyion. This concept of tripartite micelles was original, described until now only in one article (Yessine et al., 2007): a diblock cationic copolymer, a double-hydrophilic copolymer and an oligonucleotide. We need a possible disassembly at acidic pH (mimicking endosomal compartments), that is why a copolymer with a weak polyacid block is used: a polymethacrylic acid-*b*-polyethylene oxide (PMAA-*b*-POE). Moreover, in order to optimise the control of the micelle size and to confer higher stability, the choice of the copolymer is oriented towards an asymmetric polymer with block lengths of 5000 and 2100 g/mol for the POE and the PMAA blocks, respectively (Voets et al., 2007; Hofs et al., 2007). The proposed micellisation partners are of different nature: an amino acid, poly-L-lysine, and an oligosaccharide, an oligochitosan. The behaviour and the stability of the two different micelles containing either the poly-L-lysine or the oligochitosan as a function of the pH and the ionic strength will be discussed for the design of a future drug delivery system (Boudier et al., 2009b).

2. Materials and methods

2.1. Materials

The polymethacrylic acid-*b*-polyethylene oxide copolymer ($PMAA_{2100}$-*b*-POE_{5000}, $pK_a = 5.5$) was obtained from Polymer Source Inc. (USA). Water was purified with a Milli-Q purification system Millipore (France). All analytical grade reagents and chemicals including poly-L-lysine (PLL) (molecular weight between 15,000 and 30,000 g/mol, $pK_b = 10.5$) and oligochitosan (molecular weight less than 5000 g/mol, deacetylation degree >90%, $pK_c = 6.6$) were purchased from Sigma–Aldrich (France) (Fig. 1).

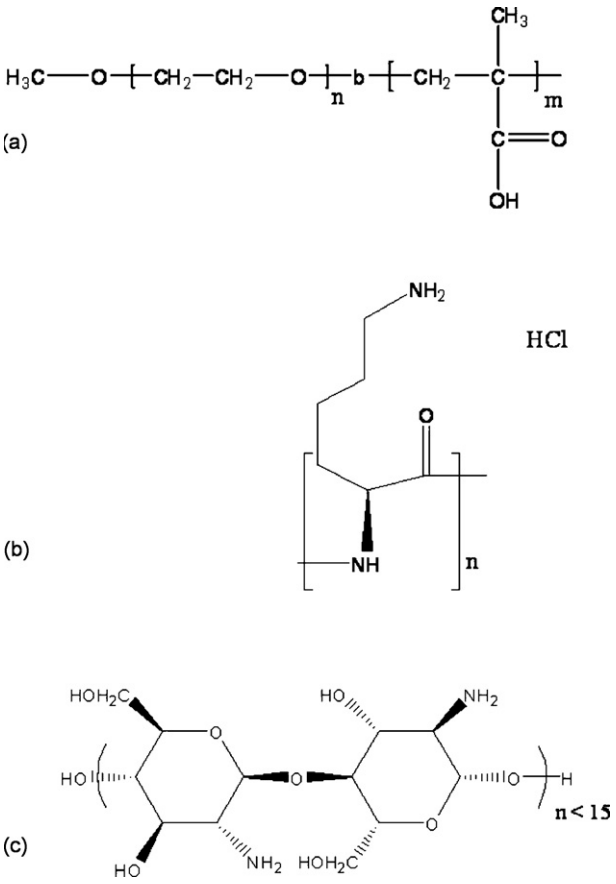


Fig. 1. Formulas of the partners used in the study (a: $PMAA_{2100}$-*b*-POE_{5000}, b: PLL, c: oligochitosan).

2.2. Micelle formation

Two different aqueous (Milli-Q water) solutions were prepared: first, the copolymer ($PMAA_{2100}$-*b*-POE_{5000}) solution at 1.3 mg/mL, secondly the counter-polyion solution at 0.73 mg/mL for poly-L-lysine (PLL) or 1.1 mg/mL for oligochitosan. The micelles were obtained by mixing the same volumes of the two solutions, corresponding to a molar ratio between amine and carboxylic acid functions of 1 ($R = [NH_2]/[COOH] = 1$) and the pH was then adjusted to 7.5 for PLL or 6.5 for oligochitosan. The final suspension was then stirred at room temperature for 3 h on a wheel shaker. The samples were stored at 4 °C until their use. For a stability study, micelles were maintained 40 days at 4 and 22 °C, then, their size and their homogeneity were assessed by dynamic light scattering (DLS).

2.3. Micelle characterisation

2.3.1. Dynamic light scattering

The analyses by dynamic light scattering (Malvern 4800, Malvern Instrument, UK) were performed with a laser having a wavelength of 532 nm, at a scattering angle of 90° and at a temperature of 22 °C. The signal to noise ratio was around 10. The average scattered intensity was measured by static light scattering. In series of samples, the scattered intensity was corrected for each volume modification of the sample. The autocorrelation function was modelled by using the CONTIN algorithm. The volume-averaged values of the Dh are given in this study.

2.3.2. Laser-Doppler electrophoresis

Laser-Doppler electrophoresis measurements were carried out in triplicate by Laser Doppler Velocytometry (NanoZS, Malvern Instrument, UK). From the obtained electrophoretic mobility, the zeta potential (ζ) was calculated using the Smoluchowski equation as follows:

$$\zeta = \frac{4\pi\eta u}{\varepsilon},$$

where u is the electrophoretic mobility, η is the viscosity of the solvent and ε is the dielectric constant of the solvent.

2.4. Counter-polyion/copolymer ratio influence on the micelle formation

Scattered intensities of $PMAA_{2100}$-*b*-POE_{5000}/PLL mixture samples or $PMAA_{2100}$-*b*-POE_{5000}/oligochitosan samples with molar ratios $[NH_2]/[COOH]$ ranging from 0.25 to 3 were determined by light scattering after 3 h stirring. The pHs were adjusted at pH 7.5 for PLL samples and 6.5 for oligochitosan-based samples. Moreover, on each sample, the zeta potential measurement was performed by Laser-Doppler electrophoresis.

2.5. pH and ionic strength influence on the micelle formation

The copolymer alone or the micelle solutions prepared according to the classical protocol (ratio $[NH_2]/[COOH]$ of 1, 3 h stirring) were studied in media with different pH or ionic strengths. pH was varied from 3.0 to 11.5, by adding NaOH (0.01 mol/L) or HNO_3 (0.05 mol/L) solutions. Ionic strength values ranging from 0 to 0.5 mol/L were assessed by adding specific volumes of NaCl solution (2 mol/L) to the samples (pH set at 7.5 for PLL micelles and 6.5 for oligochitosan micelles). Scattered intensities and hydrodynamic diameters of the samples were determined by light scattering.

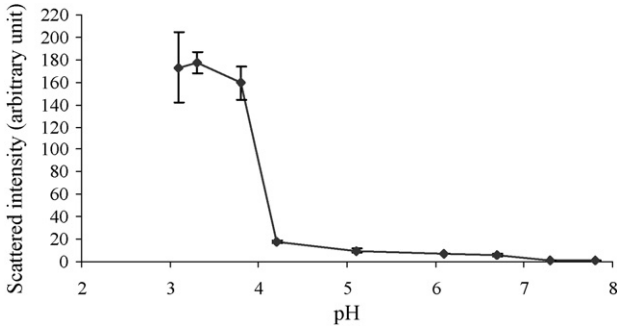


Fig. 2. PMAA2100-*b*-POE5000 copolymer behaviour as a function of the pH studied by DLS ($n = 2$).

3. Results and discussion

3.1. Copolymer $PMAA_{2100}$-*b*-POE_{5000} behaviour

First of all, the copolymer behaviour as a function of the pH and the ionic strength has to be investigated. The pH of aqueous solutions of the copolymer was adjusted at different values and solutions were analysed by DLS (Fig. 2). $PMAA_{2100}$-*b*-POE_{5000} was perfectly soluble from high pH (pH 7.8) down to pH 4.2, as shown by the low values of the light scattered intensity. Therefore, the use of the copolymer in physiological solutions (pH 7.4) is possible. On the contrary, below pH 4, a strong increase of the scattered intensity was observed, suggesting a polymer aggregation. Indeed, intra- and intermolecular hydrogen bonds between the carboxylic groups of the PMAA and the ethylene oxide units are classically described in literature (Holappa et al., 2003).

Then, $PMAA_{2100}$-*b*-POE_{5000} solutions were analysed by DLS after modification of the ionic strength from 0 to 1.5 mol/L using NaCl (Fig. 3). No aggregation of the copolymer was observed up to a concentration of 0.4 mol/L of salt, as shown by the low values of the scattered intensities. As a consequence, $PMAA_{2100}$-*b*-POE_{5000} is perfectly soluble at physiological ionic strength (corresponding to NaCl concentration of 0.15 mol/L). But, above 0.4 mol/L of salt, the scattered intensity strongly increases, revealing that NaCl diminishes the copolymer solubility due to POE dehydration. This phenomenon was already detailed in literature (Solomatin et al., 2003) leading to reverse micelles composed of a poor soluble core made of POE and salt, surrounded by a perfectly soluble PMAA corona. So, the $PMAA_{2100}$-*b*-POE_{5000} copolymer is well soluble in water at pH above 4 and at an ionic strength below 0.4 mol/L NaCl.

3.2. Micellisation principle: choice of the counter-polyion

The micelle preparation method relies on a simple mixing procedure of the $PMAA_{2100}$-*b*-POE_{5000} (Fig. 1a) and the counter-polyion.

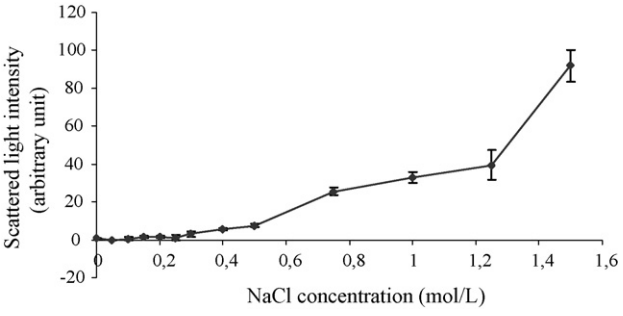
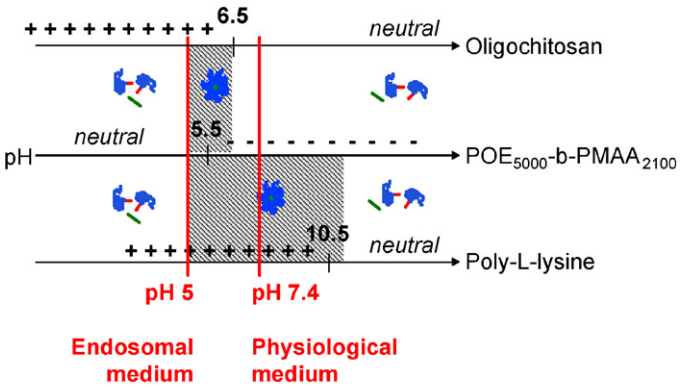


Fig. 3. PMAA2100-*b*-POE5000 copolymer behaviour as a function of the ionic strength studied by DLS ($n = 2$).



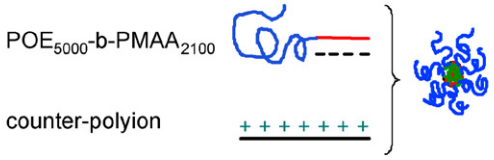


Fig. 4. Micellisation principle.

The weak polyacid block has a pK_a of 5.5 and the weak polybases have a pK_b of 10.5 (poly-L-lysine, Fig. 1b) or pK_c of 6.5 (oligochitosan, Fig. 1c). The association is induced by charge neutralization (Fig. 4) of the charged part of the copolymer with the counter-polyion leading to the formation of a coacervate surrounded by a stabilising corona (Cohen Stuart et al., 1998; Van Der Burgh et al., 2004). The polyion complex growth remains limited due to the high solubility of the POE block in the aqueous phase.

Each part of the partners plays a role in the physicochemical properties of the final micelles. Micelles will be used in physiological conditions: pH 7.4, 37 °C and at an ionic strength of 0.15 mol/L (Fig. 4). Thus, the polymethacrylic acid part of the copolymer and the counter-polyion bring the pH-sensitivity to the system, and allow the micelle to be stable at physiological pH and to disassemble under acidic pH. With regard to the temperature and the ionic strength, one cannot foresee whether the system will be stable or not. Especially it is well-described that polyion complexes are usually unstable in salty solutions (Riess, 2003). As a consequence, the stability in physiological ionic strength conditions will be precisely studied, since it is an essential criteria for the development of pharmaceutical applications.

3.3. Influence of the counter-polyion/copolymer ratio on the micelle formation

The charge ratio between NH_2 groups brought by the oligochitosan or the poly-L-lysine, and the COOH functions of the PMAA block is an important parameter in the micelle formulation. The influence of the counter-polyion/copolymer ratio, $R = [NH_2]/[COOH]$, on micelle formation was studied by DLS (Fig. 5) and by zeta potential measurements (Fig. 6) for the two kinds of counter-polyions.

As observed by DLS (Fig. 5), the poly-L-lysine samples show a plateau of stability between ratios 0.75 and 1.125. In this range of R values, high scattered intensities are obtained. Above a $[NH_2]/[COOH]$ ratio of 1.25, the excess of poly-L-lysine is not favourable to the formation of micellar objects. Dissociated polymers or small soluble complexes are present in the solution, and this is the case also below $R = 0.5$.

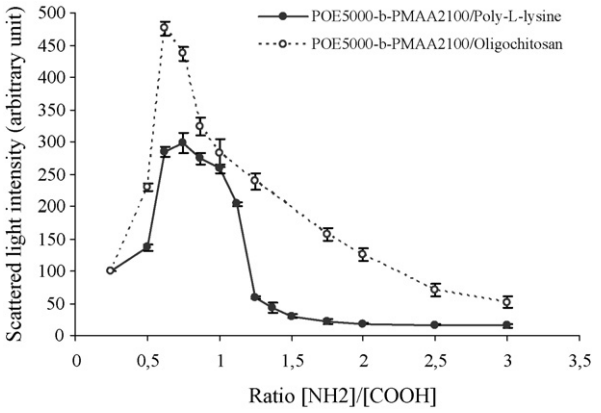


Fig. 5. Normalised scattered intensity as a function of the $[NH_2]/[COOH]$ ratio ($n = 3$).

In the case of the oligochitosan, the micelle stability is observed between $[NH_2]/[COOH]$ ratios of about 0.5 and 1. Above and under those values, the same comments as above can be done.

Also, the scattered intensity difference between the two series of samples may be explained by higher hydrodynamic diameters obtained with the oligochitosan compared to those with PLL: this will be mentioned again later in Section 3.4.

Furthermore, zeta potential measurements were performed for ratios varying from 0.25 to 5 (Fig. 6). For a $[NH_2]/[COOH]$ ratio less than 1, the copolymer was in excess, thus the average zeta potential was negative because of excess of negative charges of the polymethacrylate block. For a charge ratio corresponding to equimolarity, micelles were almost neutral: the zeta potential equals −6.1 mV for PLL samples and −4.6 mV for oligochitosan samples. Above $R = 1$, oligochitosan-based samples remained at the neutral value whereas samples with poly-L-lysine complexes showed an increasing value of the zeta potential up to +12 mV. This difference between the two polyamines might be explained by the fact that the poly-L-lysine molar mass is very high (between 15,000 and 30,000 g/mol) compared to that of oligochitosan (less than 5000 g/mol). So, excess free poly-L-lysine polymers were detected and give a positive contribution to the average zeta potential. On the contrary, the smaller oligochitosan seems to give a negligible contribution to the zeta potential in the presence of the polyion complex micelles.

3.4. Micelle size and long-term stability

The micelle characterisation results by DLS presented in Table 1 were obtained for a charge ratio of 1 and a pH of 7.5 for poly-L-lysine and 6.5 for oligochitosan. These values of the pH correspond to the domains between the pK_a and pK_b and pK_a and pK_c, respectively (Fig. 4).

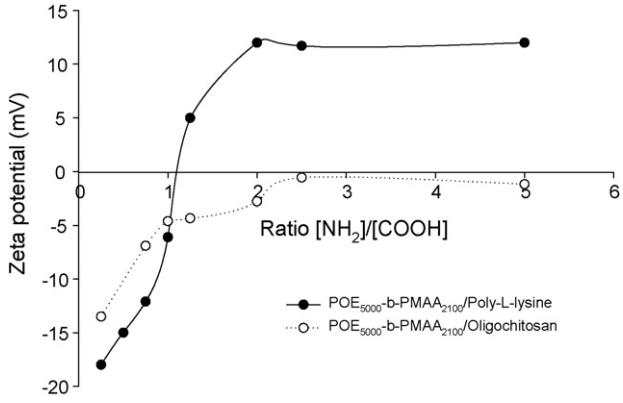


Fig. 6. Zeta potential values as a function of the $[NH_2]/[COOH]$ ratio.

Table 1
Micelle characterisation at a charge ratio of 1 and at a pH of 6.5 for oligochitosan and 7.5 for poly-L-lysine at day 1 (D1) and day 40 (D40) at 22 °C ($n = 3$, data shown as mean ± standard deviation).

Counter-polyion	Hydrodynamic diameter (nm) (mean ± sd)		Polydispersity index	
	D1	D40	D1	D40
Poly-L-lysine	28 ± 3	28 ± 2	0.20 ± 0.02	0.20 ± 0.03
Oligochitosan	60 ± 3	60 ± 3	0.20 ± 0.03	0.20 ± 0.04

Micelles have a hydrodynamic diameter of a few tens of nanometers (30 and 60 nm for poly-L-lysine and oligochitosan respectively). These results can be compared to what is usually described in literature (Riess, 2003; Bouyer et al., 2006). The small size of PLL-based micelles compared to oligochitosan micelles may be explained by the higher charge density in PLL compared to oligochitosan, leading to a denser and smaller core and then to a smaller aggregation number in the micelle. The dense PLL/DHBC core of the micelles is also due to additional interactions in the core due to the PLL properties. Indeed PLL chains inside the core adopt an alpha helix conformation and develop hydrogen bonds (Boudier et al., 2009a). Moreover, as described in the literature, the flexibility of PLL is larger than that of the chitosan and that leads to more compact aggregates when complexed with a polyanion (Maurstad et al., 2003). It is important to note that in our specific case, the flexibility of PLL induces a condensed core when micelles are formed. Also, PLL chainsin the complex micelles adopt an alpha helix conformation and that conformation is quite dense too. Size distributions of DHBC based complex micelles are most often narrow with polydispersity indices evaluated at a maximum of 0.2 whatever the counter-polyion considered. As followed by DLS every two days, oligochitosan or poly-L-lysine micelles remained stable for 40 days at 4 °C or 22 °C, without modification of neither the hydrodynamic diameter nor the polydispersity index.

A similar study was reported in the literature with polymers of higher molar masses: PMAA-*b*-POE (15,500–7500 g/mol) and poly-L-lysine (8000 g/mol). The authors described the same results obtained by DLS: the intensity-averaged hydrodynamic diameter was 140 nm with a polydispersity index around 0.1 (Li et al., 2003). Moreover, at a ratio of 1, the zeta potential was neutral and the authors suggested that the complex formation was stoichiometric. In the following physicochemical studies, a $[NH_2]/[COOH]$ ratio of 1 is chosen in order to have a neutral micelle, and a minimal quantity of excess free polymers in solution.

Thus, the present physicochemical study, which aimed at comparing two counter-polyions complexed with the polymethacrylic acid-*b*-polyethylene oxide copolymer, shows similar results. The hydrodynamic diameters equal a few tens of nanometers, the polydispersity indices are low, and the zeta potentials for a charge ratio of 1 are close to neutrality, what is usually described for amphiphilic (Kataoka et al., 2001) or double-hydrophilic block copolymer micelles (Kataoka et al., 1998). These parameters and specifically the value of the size and the homogeneity of the population are of great interest for pharmaceutical applications.

3.5. Micelle behaviour as a function of the pH and the ionic strength

Micelles were formulated in water only, without any organic solvent, in order to respect the requested conditions of pharmaceutical applications. Their stability has to be tested as a function of the pH as well as of the ionic strength.

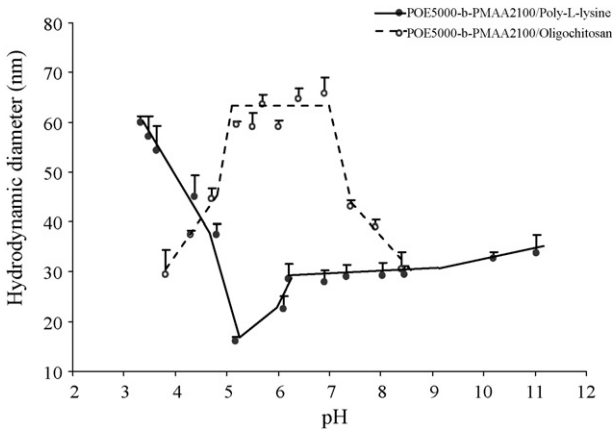


Fig. 7. Hydrodynamic diameters of micelles as a function of the pH ($n = 3$).

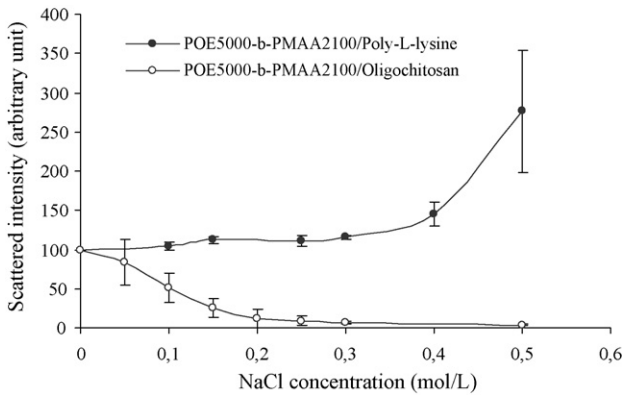


Fig. 8. Normalised scattered intensity as a function of NaCl concentration ($n = 3$).

3.5.1. Micelle behaviour as a function of the pH

The comparative pH sensitivity study between oligochitosan and poly-L-lysine micelles is presented in Fig. 7. In these experiments, micelles were first formed at a pH close to 7 (pH 6.5 for oligochitosan and pH 7.5 for PLL) and then the pH of the solution was adjusted at the desired value from pH 3 to 11. Results obtained with oligochitosan micelles showed stability between pH 5 and 7 with a volume-averaged hydrodynamic diameter of 60 nm, whereas poly-L-lysine micelles were stable from pH 6.5 to basic pH (pH 11) with a volume-averaged hydrodynamic diameter of 28 nm.

Under acidic pH, pH < 6 for PLL and 5 for oligochitosan, a disassembly was observed for the two kinds of counter-polyions due to the decrease of the ionisation degree of the copolymer. For poly-L-lysine micelles, the hydrodynamic diameter increased below pH 5, which can be explained by the copolymer aggregation as evaluated in Fig. 2. This phenomenon could be observed with oligochitosan micelles if the experiment was continued towards more acidic pH. Moreover, for the two polyamines, the micelle disassembly starts at a different pH, this is because the polymer p*K* is a parameter which varies as a function of the presence of a counter-polyion as well as of the nature of the counter-polyion (Goloub et al., 1999). At basic pH, pH > 7, oligochitosan micelles disassembled because of the decrease of the ionisation degree of this compound. For the particular case of poly-L-lysine, micelles remained stable even under very basic conditions, up to pH 11, this is due to the high value of the pK of the PLL. Moreover, a recent work published by our group assessed that hydrogen bonds as well as hydrophobic domains develop within the core of those micelles (Boudier et al., 2009a,b). As a conclusion, the two types of micelles disassembled under acidic conditions but the poly-L-lysine micelles were the only ones to remain stable at the physiological pH 7.4.

3.5.2. Micelle behaviour as a function of the ionic strength

The behaviour of the micelles faced with an increased ionic strength was studied by DLS and the results are shown in Fig. 8. The micelles needed for biological applications have to be stable at the physiological salt concentration of 0.15 mol/L. For oligochitosan micelles, the increase of ionic strength induced a scattered intensity drop suggesting the micelle disassembly. This is due to charge screening of the polyions of the copolymer and the oligochitosan (constituting the micelle core) with sodium and chloride ions (Cölfen, 2001). This observation confirms that the formation of oligochitosan based micelles results from electrostatic interactions only. On the opposite, poly-L-lysine micelles showed a stable scattered intensity despite the increase of the salt concentration up to 0.4 mol/L of NaCl. This result shows that the organisation of the poly-L-lysine micelle core does not allow salt to dissociate the polymers in the core. The stability of poly-L-lysine based micelles against salt confirms that PLL based micelle formation results from different kinds of interactions. Indeed, a study by circular dichroism and by spectrofluorimetry using pyrene revealed that the micellisation process is parallel to hydrogen bondings and to hydrophobic domain development within the core of the objects (Boudier et al., 2009a). Moreover, as shown in Fig. 2, the increase of the scattered intensity above 0.5 mol/L of salt was explained by the copolymer specific behaviour under high salt concentration, i.e. POE solubility decreases at high NaCl concentration. As a conclusion of this part, poly-L-lysine micelles are the only ones that can be used for the above-mentioned application in the pharmaceutical field.

To conclude on the micelle behaviour as a function of pH and ionic strength, two different behaviours were revealed as a function of the counter-polyion. Indeed, although oligochitosan-based micelles are stable in the pH range between 5 and 7 and present an efficient pH-sensitivity, they demonstrated no stability under physiological ionic strength. The high salt concentration induces micelle destabilisation due to charge screening in the micelle core. Indeed it is classically described that electrostatic interactions inducing polyion complex micelle formation do not resist to high salt concentration. Actually, the main driving force for micelle formation is the entropy gain resulting from the release of small counterions initially bound to the charged part of the polyelectrolytes. This small ion departure is parallel to the development of the electrostatic interactions between the copolymer and the counter-polyion (Riess, 2003). This argument explains the large sensitivity of such systems to ionic strength variation. Thus salt and therefore small ion addition decreases electrostatic interactions and then induces micelle disassembly. Actually, this phenomenon must be a problem for drug encapsulation and as a consequence for pharmaceutical applications (Bronich et al., 1999). This problem can be overcome by the chemical modification of the compounds or by a relevant choice of the counter-polyion. Thus in the specific case of the poly-L-lysine-based micelles, all the parameters needed for pharmaceutical formulation were checked: pH-sensitivity under acidic conditions as well as good stability in physiological-like media and lastly excellent stability with time.

4. Conclusion

This study has underscored the importance of the choice of the partners in the specific case of double-hydrophilic block copolymer complex micelles for pharmaceutical applications. Thus, it was confirmed that PLL complexes with PMAA-*b*-POE copolymers present a behaviour, which is well adapted to pharmaceutical applications. These micelles thanks to their physicochemical properties appear as potential interesting drug delivery systems.

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