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The control of dendritic cell maturation by pH-sensitive polyion complex micelles

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ABSTRACT

Double-hydrophilic block copolymer micelles were designed as vectors for *ex vivo* dendritic cell engineering to improve the delivery of therapeutic molecules in such immune cells. Polymethacrylic acid-b-polyethylene oxide ($PMAA_{2100}$-b-POE_{5000})/poly-L-lysine micelles were optimised and showed a hydrodynamic diameter of 30 nm with a peculiar core organised with hydrogen bonds as well as hydrophobic domains. The micelles proved high stability in physiological conditions (pH and ionic strength) and were also able to disassemble under acidic conditions mimicking acidic endolysosomes. The efficient endocytosis of the optimised micelles tested on bone marrow-derived dendritic cells was monitored by fluorescence-activated cell sorting and microscopy analysis. Finally, the micelle biocompatibility permitted a complete control of the dendritic cell-maturation process widening the therapeutical potential of such engineered dendritic cells for cancer vaccines as well as for immunomodulation in autoimmune diseases.

1. Introduction

The controlled synthesis of nanomaterials has received much attention in recent years in many fields [1] and particularly in drug formulation [2]. Core–shell micelles made of amphiphilic block copolymers have been widely studied by researchers because of their ability to encapsulate lipophilic drugs. Thus, paclitaxel, an anticancer drug, was formulated with poly(ethylene glycol)-b-poly(-D-L-lactic acid) [3], and cyclosporine used to prevent organ rejection after transplantation with poly(ethylene oxide)-b-(poly-ϵ-caprolactone) [4]. The micellar objects present a narrow size distribution centred on a few tens of nanometers, which prevents their uptake by the reticuloendothelial system and facilitates the targeting of inflammatory or cancer tissues [5]. More recently, core–shell double-hydrophilic block copolymer-based micelles have been fabricated. The core formation is often the result of electrostatic interactions between one hydrophilic charged block of the copolymer and an oppositely charged counter-polyion creating an insoluble coacervate in aqueous solution [6,7]. The second hydrophilic block of the copolymer allows the formation of well-defined entities remaining in solution, due to the steric stabilisation brought by the corona of neutral polymer blocks. Many studies have already led to formulate micelles with a biocompatible polyamino acid-b-polyethylene oxide copolymer and oppositely charged species such as cisplatin [8], doxorubicin [9], peptides or proteins as vasopressin [10] or lysozyme [11], and also nucleic acids [8].

The advantage of double-hydrophilic block copolymer (DHBC) micelles compared with the amphiphilic block copolymer ones, besides their simple preparation [3], lies on their interesting ability to be responsive to environmental *stimuli* such as pH, ionic strength or temperature variation [2]. This property makes them attractive as drug vector for the targeting not only of tissues, classically described in the case of amphiphilic micelles because of the presence of leaky capillaries [5], but also of intracellular organites and particularly acidic endolysosomes created after cell endocytosis [8]. In this way, application of polyion complex micelles as pH-sensitive systems in cell therapy using dendritic cells (DCs) may offer a new powerful therapeutical approach in vaccines as well as in autoimmune diseases [12]. Indeed, those cells are the most potent antigen-presenting cells able to initiate primary immune responses as well as to restore tolerance to self-antigens [13]. Thus DCs present peptides from pathogens or self-antigens linked with major histocompatibility complex (MHC) class I or class II molecules. The presentation of such processed peptides to T cells could lead to their activation depending on the nature of the peptide, the maturation state and the cytokine-secretion profile of the DCs as well as the

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microenvironment. The maturation or activation state of the DCs seems to play one major role in the initiation of the adaptive immune response since the fully mature DCs (mDCs) are the most potent antigen-presenting cells able to prime naive T cells. In the opposite, the immature DCs (iDCs) exert a sentinel function and may induce tolerance, even if such paradigm has recently been challenged in the literature [14]. These tolerogenic DCs have been shown to induce regulatory T cells that can suppress an already primed immune response and reverse autoimmunity in several experimental models. The challenge of such a therapeutic approach is to deliver a molecule into the cytosol without inducing the maturation process of the DCs, which are sensitive to any stress signal. The aim of this work was, first, to formulate double-hydrophilic block copolymer micelles, to characterise them with classical physicochemical methods and to check the stability of the optimised micelles as a function of environmental parameters (pH, ionic strength) and in the cell culture medium. In the last part, the interaction of such vectors with dendritic cells was evaluated in terms of cytotolerance, endocytosis and phenotypical changes.

2. Materials and methods

2.1. Materials

The polymethacrylic acid-b-polyethylene oxide copolymer ($PMAA_{2100}$-b-POE_{5000}) was obtained from Polymer Source Inc. (USA) and water was purified with a Milli-Q purification system Millipore (France). BalB/C mice were purchased from Harlan (France), mouse pan T and pan B Dynabeads from Dynal Biotech (France) and recombinant murine GM-CSF and recombinant murine IL-4 were obtained from R&D Systems (France). Fluorescein isothiocyanate labelled MHC class II antibody (FITC-anti-MHCII) was purchased from Miltenyi Biotec (France) and Phycoerythrine-anti-CD86 (PE-anti-CD86), PE-anti CD11c and PE-anti CD40 from BD Biosciences (France). All analytical reagents grade chemical including poly-L-lysine (PLL) and fluorescein isothiocyanate labelled poly-L-lysine (FITC-PLL) (molecular weight between 15,000 and 30,000 g/mol) and buffer components were purchased from Sigma Aldrich (France). All reagents used to maintain cells were obtained from Invitrogen (France).

2.2. Micelles formation and stability

For the micelle synthesis, two Milli-Q water solutions were prepared at 1.3 mg/mL of polymethacrylic acid-b-polyethylene oxide copolymer ($PMAA_{2100}$-b-POE_{5000}) (pK_a 5.5) and 0.73 mg/mL of poly-L-lysine (PLL) (pK_b 10.5). The micelles were obtained by mixing the same volumes of the two polymer solutions, corresponding to a molar ratio between amine and carboxylic functions of 1 ($R = [NH_2]/[COOH] = 1$) and the pH was then adjusted to 7.5, which is in the micelle stability domain between the two pKs of the compounds. A similar micelle solution was prepared from polymer solutions first dissolved in phosphate buffer solution, PBS (sodium chloride 4 g, disodium phosphate dihydrated 0.575 g, monopotassium phosphate 0.1 g, potassium chloride 0.1 g for 500 mL Milli-Q water, at pH 7.4) for fluorescence measurements and for the cellular study. The final suspension was then stirred at room temperature for 3 h. The samples were stored at 4 °C until they were used. Their stability was investigated using micelles prepared according to classical protocol in two different studies: (i) as a function of the pH, the pH values were adjusted from 3.0 to 11.5 with NaOH (0.01 M) or HNO_3 (0.05 M) solutions or (ii) as a function of the ionic strength from varying the NaCl concentration from 0 to 0.5 M.

2.3. Dynamic light scattering

Scattered intensities and object sizes of suspension of micelles and $PMAA_{2100}$-b-POE_{5000}/PLL mixtures with molar ratio $[NH_2]/[COOH]$ ranging from 0.25 to 3 were determined by Dynamic Light Scattering (Malvern 4800, Malvern Instrument, UK). The analysis was performed with a laser wavelength of 532 nm, scattering angle 90° and at a temperature of 22 °C.

During the dynamic light scattering measurements, the autocorrelation function was developed by the following equation:

$$g^{(1)}(\tau) = \exp\left[-\Gamma\tau + (\mu_2/2)\tau^2 - (\mu_3/3!)\tau^3 + \ldots\right], \quad (1)$$

yielding an average characteristic line width of Γ. The z-averaged diffusion coefficient was obtained from Γ based on the following equations:

$$\Gamma = Dq^2, \quad (2)$$

$$q = (4\pi n/\lambda)\sin(\theta/2), \quad (3)$$

where q is the magnitude of the scattering vector, n is the refractive index of the solvent, λ is the wavelength of the incident beam, θ is the detection angle, and D is the diffusion coefficient. The hydrodynamic diameter d_h can then be calculated using the Stokes–Einstein equation:

$$d_h = k_b T/(3\pi\eta D), \quad (4)$$

where k_b is the Boltzmann constant, T is the absolute temperature, and η is the viscosity of the solvent. Also, the polydispersity index $PI = \mu_2/\Gamma$ was derived from Eq. (1).

The scattered intensity was corrected for each volume modification. The autocorrelation function was modelled by the CONTIN algorithm. The volume-averaged values of the d_h are given in this study.

2.4. Laser-Doppler electrophoresis

Laser-Doppler electrophoresis measurements of the samples at pH 7.4 (micelles and $PMAA_{2100}$-b-POE_{5000}/PLL samples with a $[NH_2]/[COOH]$ molar ratio ranging from 0.25 to 3) 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:

$$\zeta = (4\pi\eta u)/\varepsilon,$$

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

2.5. Circular dichroism

PLL solutions and micelles at various pH ranging from 5.3 to 10.5 and on the other hand, $PMAA_{2100}$-b-POE_{5000}/PLL samples with a $[NH_2]/[COOH]$ molar ratio ranging from 0.25 to 3, were analysed by circular dichroism using a Chirascan (Applied Photophysics, UK) with the soft ProData Chirascan (4.0), with a xenon lamp and with a 0.5-mm cell. The measurement was carried out in triplicate. Molar ellipticity, $[\theta]$, was calculated according to the following equation:

$$[\theta] = \theta/(CIN_p),$$

where $[\theta]$ is in deg cm^2 dmol^{-1}, θ is the specific ellipticity in deg, C the poly-L-lysine concentration in mol cm^3, l the cell length in dm and N_p the number of residues. Moreover, alpha helix percentage calculation was performed according to Greenfield and Fasman [15].

2.6. Fluorescence measurements

2.6.1. Polarisation experiments

Samples with FITC-PLL/$PMAA_{2100}$-b-POE_{5000} molar ratio ranging from 0.25 to 3 were prepared. The samples were illuminated with vertically polarised light and the components of the emitted intensity, respectively polarised vertically ($I_\parallel$) and horizontally ($I_\perp$), with the reference to the direction of the excitation light, were measured successively for each sample. The excitation and emission wavelengths were set to 490 and 520 nm respectively, with both 3 nm bandwidths. The steady-state fluorescence polarisation factor p was determined according to the following equation:

$$p = \left(I_\parallel - GI_\perp\right)\Big/\left(I_\parallel + GI_\perp\right)$$

where G is the compensating factor for the anisotropic sensitivity of the instrument (0.64 in our experimental case). All measurements were carried out in duplicate.

2.6.2. Hydrophobicity measurements

Pyrene was first dissolved in methanol at a concentration of 1.9×10^{-3} mol/L and then diluted in Milli-Q water to obtain a concentration of 1.9×10^{-5} mol/L. 50 µL of this solution was added to each mixture composed of 1 mL of $PMAA_{2100}$-b-POE_{5000}/PLL dissolved in PBS with $R = [NH_2]/[COOH] = 1$ and adjusted at various pH. Pyrene was also used to determine the critical association concentration of the system with the use of various concentrations of micelles corresponding from 19.5 to 6.5×10^{-5} mg/mL of copolymer. The pyrene emission spectra were recorded after 30 min of incubation using an excitation wavelength of 340 nm. The emission and excitation slit widths were set at 5 and 3 nm.

All the fluorescence measurements were performed on an RF 5302 Shimadzu spectrofluorometer (Japan) equipped with a xenon light source (UXL-150S, Ushio, Japan) and were carried out in a 1×1 cm-path length quartz cuvette (Hellma, Germany).

2.7. Dendritic cell

The immature and mature dendritic cells (iDCs and mDCs respectively) were obtained from BalB/C mice according to Inaba as previously described [16]. Briefly, bone marrow cells were harvested from the femurs and tibiae of BalB/C mice and washed in RPMI 1640 following lysis of red blood cells. T and B cells were depleted using mouse pan T and pan B Dynabeads, and monocytes were removed by adhesion