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

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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 (PMAA₂₁₀₀-b-POE₅₀₀₀) / 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 organelles 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 micro-environment. 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 cytotoxicity, endocytosis and phenotypical changes.

2. Materials and methods

2.1. Materials

The polymethacrylic acid *-b-* polyethylene oxide copolymer (PMAA₂₁₀₀-*b-*POE₅₀₀₀) 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 Phycoerythrin-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 15000 and 30000 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 (PMAA₂₁₀₀-*b-*POE₅₀₀₀) (pKa 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 ($R = [\text{NH}_2]/[\text{COOH}] = 1$) 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 three hours. 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 HNO₃ (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 PMAA₂₁₀₀-b-POE₅₀₀₀/PLL mixtures with molar ratio $[\text{NH}_2]/[\text{COOH}]$ 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:

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

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:

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

where k_b is the Boltzmann constant, T is the absolute temperature, η is the viscosity of the solvent.

Also, the polydispersity index ($PI = \mu_2 / \bar{r}^2$) 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 PMAA₂₁₀₀-b-POE₅₀₀₀/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:

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

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, PMAA₂₁₀₀-b-POE₅₀₀₀/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:

$$[\theta] = \theta / (C l N_p),$$

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

2.6. Fluorescence measurements

Polarisation experiments

Samples with FITC-PLL/PMAA₂₁₀₀-b-POE₅₀₀₀ 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 ρ was determined according to the following equation:

$$\rho = (I_{\parallel} - G I_{\perp}) / (I_{\parallel} + G I_{\perp})$$

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.

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 μ L of this solution was added to each mixture composed of 1 mL of PMAA₂₁₀₀-b-POE₅₀₀₀/PLL dissolved in PBS with $R=[\text{NH}_2]/[\text{COOH}]=1$ 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 a RF 5302 Shimadzu spectrofluorometer (Japan) equipped with a Xenon light source (UXL-150S, Ushio, Japan) and were carried out in a 1 x 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 by 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 in RPMI 1640 5% Foetal Calf Serum (FCS). Remaining cells were cultured in complete medium (RPMI 1640 supplemented with 5% FCS, 2 mM L-glutamin, 5×10^{-5} M β -mercaptoethanol, 100 U/mL penicillin, 100 μ g/mL streptomycin, 0.1 mM essential amino acids, and 1 mM sodium pyruvate) with 1 000 IU/mL recombinant murine GM-CSF and 1 000 IU/mL recombinant murine IL-4 at 5×10^5 cells/mL in a 24-well plate. Culture medium was renewed at day 2 and 4. To induce maturation

of DCs, DCs were harvested at day 6, and incubated for 24 h with 1 µg/mL of lipopolysaccharide (LPS). Micelles were coincubated with cells at a concentration corresponding of 0.13 mg/mL of copolymer.

2.8. Fluorescence-activated cell sorting (FACS)

Cells were incubated with different antibodies for 15 min in the dark according to the manufacturer specifications. Cells were washed three times with PBS/Bovine serum albumin (0.1%) / sodium azide (0.01%) before analysis on a FACScan (Becton Dickinson, USA) with Cell Quest Pro soft. For cell viability analyses, the addition of propidium iodide (1 µM) was performed just before FACS analysis.

2.9. Fluorescence microscopy

Lab-Teck slides (Nunc, Germany) were coated with poly-L-lysine (molecular weight between 30000 and 70000 g/mol) for one hour at room temperature and washed twice with sterile water and twice with RPMI 5% FCS. Cells were incubated with FITC-PLL micelles at 37°C or 4°C and washed three times. They were then adhered for 30 min at 37°C on the slides and fixed with paraformaldehyde 6 % for 15 min at room temperature. After two washings, cells were incubated with NH₄Cl 0.5 M for 15 min at room temperature and finally washed twice with PBS. Slides were mounted according to the manufacturer specifications with Vectashield mounting medium containing diamidino-2-phenylindol, DAPI (Cliniscience, France). The observations were performed using a AxioImager equipped with Apotome (Carl Zeiss, Germany).

2.10. Cytokine quantification

DC supernatants were recovered after 24h of incubation with micelles. Cytokine detection and quantification were performed using a standard sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer recommendations (eBioscience, USA).

2.11. Endotoxin quantification

The endotoxin content of micelle suspension and related solubilised compounds used was determined by chromogenic Limulus Amebocyte Lysate assay (QCL-1000, Lonza, France). The quantification was performed according to the manufacturer recommendations using a 96-well microplate format.

3. Results and discussion

3.1. Micelles formation

The micelles formation relied on a simple reproducible method and consists in mixing a double-hydrophilic block copolymer aqueous solution and a counter-polyion aqueous solution. A weak polyacid for one of the copolymer blocks was chosen because of its pKa near 5 permitting a micelle disassembly in acidic medium. For the second block, a classic polyethylene oxide was adopted. The counter-polyion was chosen as a weak polybase with a pKb around 10.5 in order to guarantee its ionised state in physiological medium to obtain stable micelles at pH 7.4 (Fig. 1). These parameters will be discussed in the stability study.

First, the micelle formation was followed as a function of the molar ratio $R=[\text{NH}_2]/[\text{COOH}]$ by measuring the scattered light intensity; the charge of the objects was obtained by electrophoretic measurements and the conformation of poly-L-lysine by circular dichroism (Fig. 2A). At R below 0.5, poly-L-lysine polymers are highly deficient compared to the copolymers in terms of charge compensation; in that range of R values, a low value of the scattered light intensity and a highly negative zeta potential (-15 mV) of the suspension are obtained. These results show that, in this range of R values, polymers are not associated. Micelles are absent below $R=0.5$, and this is in agreement with the observation of a very low alpha helix percentage of the PLL chains, as determined by circular dichroism and calculated according to Greenfield [15]. Indeed, free PLL chains are under an elongated conformation at pH=7.5. From $R=0.5$ to 1, the light scattered intensity considerably increases and becomes maximal around a molar ratio between amino and carboxylate groups of 1. This shows that polymers aggregate to form bigger objects. A hydrodynamic diameter D_h of the colloids of 30 nm is obtained for $R=1$. Furthermore, a polydispersity index of 0.2 was measured, suggesting a rather narrow size distribution. At $R=1$, the charge compensation between acidic and basic functions is expected to be optimal, and an insoluble electrostatic complex between the two blocks can form. Well-defined micelles are then obtained because POE blocks are able to stabilise the coacervate between the two charged components (PMAA and PLL) [6]. At $R=1$, the zeta potential value was nearly neutral (close to -6 mV), in agreement with the expected charge compensation and the presence of the POE corona. The percentage of alpha helix within the micelles was maximum (40%) at $R=1$ as determined by circular dichroism. This is a high value in comparison with the value obtained when poly-L-lysine is

free in solution at pH 7.4, (only 10 % or so of alpha helix are observed) but this feature has already been observed when PLL is present in the core of DHBC-based micelles. Our results are in accordance with previous studies reported on micelles formed with poly-L-lysine with a molecular weight larger than 8000 g/mol and a PMAA-b-PEO with block molecular weights of 15500 and 7500g/mol respectively [17], as well as previously published results using poly-L-lysine-g-poly(lactic-co-glycolic acid) and DNA [18]. When R increases from 1 up to 1.5, the scattered light intensity decreased sharply, together with the alpha helix percentage, which went down from 40% to 10%. That decrease may be the signature of free PLL in solution. These changes are accompanied by a change in the zeta potential from an almost neutral value to 13 mV. At high R ratio, poly-L-lysine polymers are in excess compared to the PMAA₂₁₀₀-b-POE₅₀₀₀ copolymers and the formation of micelles seems to be prevented due to the high excess of positive charge.

Fluorescent labels have been classically used to study the micro-environment of polyelectrolyte complexes. Fluorescein isothiocyanate (FITC) is one of the most common labelling fluorescent reagents used, because when fixed to a molecule, its ability to move depends on the molecule movements [19]. In our measurements, the use of FITC covalently linked to poly-L-lysine allowed us to follow the micellisation process; we studied the evolution of the fluorescence polarisation p of FITC as the molar ratio $[\text{COOH}]/[\text{NH}_2]$ was increased (Fig. 2B). When the molar ratio $[\text{COOH}]/[\text{NH}_2]$ increased from 0 to 0.5, a sharp increase of p from 0.08 to 0.13 was observed, which corresponds to a decrease in poly-L-lysine motion, due to the formation of an insoluble electrostatic complex. Then, above the molar ratio $[\text{COOH}]/[\text{NH}_2]$ of 0.5, a plateau was reached at a high p value, meaning that the movements of poly-L-lysine molecules were completely blocked due to their complexation with polymethacrylate blocks. This fluorescence study reinforces previous results presented in Fig. 2A. At a molar ratio $[\text{COOH}]/[\text{NH}_2]$ of 1, all poly-L-lysine polymers are complexed to diblock polymers and the suspension only contains well-defined micelles. This composition was chosen for further experiments performed in order to characterise the complex in the core.

3.2. The micelle core

Hydrogen bonds

The conformation of poly-L-lysine, optically active, was analysed into solutions in the absence of any copolymer and then in the micelle suspension using circular dichroism. The analysis of free poly-L-

lysine in solution showed (inset graph of Fig. 3A) that, at pH below pK_b (10.5), the poly-L-lysine conformation was mostly in random coil with a maximum molar ellipticity observed at 217 nm and a minimum at 197 nm. When pH increased above the pK_b value, the conformation changed to alpha helix with a maximum at 191 nm and two minima at 208 and 222 nm, as also described by Greenfield [15]. Furthermore, the occurrence of a crossover wavelength at 204 nm, when pH varies, shows that the change in conformation from random coil to alpha helix is pH-sensitive, as mentioned in the literature [20]. Then, we characterised the conformation of poly-L-lysine within the micelles; indeed, we observed that the presence of the block copolymers induced modifications of the circular dichroism graphs as a function of the pH. We studied whether the formation of alpha helices was correlated with the formation of micelles (Fig. 3A). We showed that in the pH range where micelles are expected, ie. between pH 5.5 to 10.5, poly-L-lysine adopted an alpha helix conformation, suggesting the presence of hydrogen bonds between polymers inside the core. We compared the alpha helix percentage of poly-L-lysine free in solution to that of poly-L-lysine inside the micelle core, as a function of pH (Table 1).

The difference between free poly-L-lysine and poly-L-lysine loaded into the micelle core is notable: when pH is higher than 7, the alpha helix percentage is largely superior (49% at pH 7) in the system PLL-DHBC, to that of free poly-L-lysine in solution (10% at pH 7). At pH 5.3 the small difference between the two cases may be due to the presence of some hardly soluble complexes between PLL and the copolymer as PMAA is partially ionised. Those results can be compared to a study in which the formation of poly-L-lysine-b-polyethylene glycol and all trans-retinoic acid particles is investigated [21]. Similarly to what is obtained here, the alpha helix percentage of poly-L-lysine is high between the two pK of the components, a fact related to the formation of objects in that pH range.

Hydrophobic domains

The polarity of the micelle core was estimated using free pyrene (non covalently linked), which was used as a fluorescent probe. The spectra of the molecule and particularly the intensity of its first and third vibronic bands (I1 and I3 respectively) are a direct function of the micro-environment polarity [22, 23]. The results (Fig. 3B) firstly show that the I1/I3 ratio at any pH was lower than the value usually given for water [24] (1.87) suggesting that pyrene is localised in micro-environments less polar than water. Moreover, the I1/I3 ratio varies as a function of pH. At pH 5, the value of I1/I3, 1.18, shows that

pyrene is in a quite polar microenvironment (suggesting that micelles are disassembled at pH 5). At pH higher than 7.5, I₁/I₃ is lower, which suggests the presence of polyion electrostatic complex micelles in that pH range. Furthermore, the alpha helix percentages seem to be in accordance with the appearance of hydrophobic domains. As suggested in the literature, when PLL forms a complex with another polyelectrolyte, the energetically favorable coil-helix transition may occur and may contribute to the stabilisation of the complex [19]. A more ordered poly-L-lysine conformation may allow optimised interactions between PMAA and PLL due to the favourable positions of the PLL charges. As a consequence, the formation of a neutral complex allows the appearance of hydrophobic domains.

3.3. Critical association concentration

In order to evaluate the critical association concentration, the pyrene fluorescence in micelle suspension of different concentrations was studied since the presence of hydrophobic domains is an evidence of micelle formation. A value of 22 mg/L was determined for the present system, close to what is usually obtained with other double hydrophilic block copolymer systems [3].

3.4. Stability

As a function of the pH

The stability of the micelles was studied following the variations of the scattered light intensity and of the hydrodynamic diameter as a function of pH (Fig. 4A). A stability study as a function of pH showed a plateau of micelle formation at pH values above 6.5, with a hydrodynamic diameter measured at 30 nm. The plateau approximately corresponds to the pH range between the pK of the two components: pK_a 5.5 for PMAA₂₁₀₀-b-POE₅₀₀₀ and pK_b 10.5 for PLL. It is important to notice the good micelle stability at physiological pH, which is an essential property for the development of pharmaceutical formulations. On the other hand, when pH decreases from 6.5 down to 5, the hydrodynamic diameter and the scattered light intensity decrease, showing the dissociation of the micelles. This is an important feature for our targeted biological application, because pH 5 corresponds to the pH found in dendritic cell acidic endolysosomes [12]. As micelle disassembly occurs at pH 5, the present pH-sensitive micelles may be considered as candidates for controlled drug delivery systems within the cells after endocytosis. When decreasing pH below 5, the hydrodynamic diameter and the scattered

intensity increase, this is assigned to the fact that PMAA₂₁₀₀-b-POE₅₀₀₀ polymers start to aggregate due to intermolecular H bond formation [25].

As a function of the ionic strength

The stability of the micelles was then studied as a function of the ionic strength. The micelles were found to be stable up to a 0.4 M NaCl concentration and to present a constant hydrodynamic diameter of 30 nm (Fig. 4B); such results are different from a majority of published results on the stability of polyion complex micelles [26]. In electrostatic complex micelles, it is usually observed that micelles are dissociated at such a salt concentration. The results obtained here may be explained by the presence of PLL in alpha helix conformation as well as by the existence of hydrogen bonds, creating hydrophobic domains, which increase the stability of the complex structure. Thus, the electrostatic interactions are at the origin of the formation of the complex core but are not the only interactions responsible for the core stability. The high stability of the present micelles at physiological ionic strength (0.15 M) allowed us to consider this system for drug delivery applications in cells. A similar stability was previously observed by Govender with complex micelles of polyethylene-glycol-*b*-polyaspartic acid and diminazene in the same salt concentration range [27]. They did not observe any dissociation of the micelles despite the high salt concentration and attributed this result to the presence of hydrogen bonds. Increasing the salt concentration above 0.4 M leads to an increase of the hydrodynamic diameter of the colloids, consistent with the POE dehydration at high ionic strength. A decrease in steric repulsion between micelles may appear, leading to aggregates [26]. As a conclusion, the physicochemical investigation of the behaviour of the micelles in physiological conditions as well as in acidic solution showed that the present system may be considered as a candidate for dendritic cell therapy.

In the cell culture medium

After dilution in the complete cell culture medium, the hydrodynamic diameter and the polydispersity index of the micelles was measured respectively at 30 nm and 0.2 and remained unchanged after 3 hours of incubation, meaning that the micelles were stable in such physiological conditions. Moreover, this confirmed that micelles could be endocytosed stands and were not coupled with substances contained in this medium such as proteins or carbohydrates.

3.5. Cytotoxicity to dendritic cells

In order to validate our formulation, we evaluated the interaction of the micelles *ex vivo* with murine bone marrow-derived DCs. The micelles, as well as solubilised individual compounds alone, were added to murine bone marrow-derived DCs in order to assess their effect on such cells. After 24 hours of incubation, percentages of viable DCs were evaluated by Trypan Blue exclusion. Results showed that PLL solution was found to be highly toxic for the DCs, and led to a complete destruction of the cells. However, the copolymer PMAA₂₁₀₀-b-POE₅₀₀₀ solution was well tolerated with almost 70% of viable cells after 24h incubation. More importantly, 80% of the DCs were still alive after 24h of incubation with the micelles compared with 50% of living cells obtained after incubation with a commercial cationic lipid. The good cytotoxicity of such vector was confirmed by a more sensitive and quantitative method using a death cell marker, propidium iodide (PI) followed by fluorescence-activated cell sorting (FACS) analyses. In these experiments 12.5% of PI positive cells were obtained for iDC control versus 9.8% of PI positive cells for DCs incubated with micelles corresponding to 0.13 mg/mL copolymer solution, and 14.7% of PI positive cells for iDCs incubated with micelles corresponding to 0.013 mg/mL copolymer solution.

3.6. Endocytosis

The endocytosis efficiency was evaluated using FITC-labelled micelles by FACS. In the used *in vitro* cell culture conditions, DCs were specifically labelled with the PE-anti CD86 antibody recognising the co-stimulatory molecule whose level of expression is dependent on the maturation state of the DCs. Thus the efficiency of the endocytosis was followed by assessing the percentage of FITC-positive cells gated on CD86 positive cells (Fig. 5). In this experiment a kinetic of endocytosis was compared between iDCs and mDCs at 37°C. In order to evaluate the micelle adsorption on the DC membrane, an incubation control was performed at 4°C to block the intracellular trafficking. As previously described, the endocytosis was found to be really more efficient in immature versus mature DCs. Indeed, endocytosis is a rapid process in iDCs with 30% of FITC-positive cells within the first 10 minutes, reaching a plateau around 50% of positive cells during the first hour. This plateau could be explained by two parameters: first, a real saturation of the endocytic process and/or the delivery of the FITC-labelled micelles within the intracellular acidic compartments implying the decrease of FITC

signal while endocytosis continues. In order to confirm these results we analysed the endocytosis of FITC-labelled micelles by live cells after 3 hours of incubation (Fig. 6) by fluorescence microscope equipped with ApoTome. The cells maintained at 4°C showed significantly weaker fluorescence staining compared to those incubated at 37°C. The DCs treated with the micelles at 37°C showed multiple fluorescent intracellular staining, probably corresponding to phagocytic/endocytic compartments. These results confirm the efficient endocytosis of the PMAA₂₁₀₀-b-POE₅₀₀₀ micelles by the DCs.

3.7. Maturation control

Dendritic cells are known to be very sensitive to any stress signal that induces their maturation characterised by an increase of MHC class II and co-stimulatory molecules (particularly CD86 and CD40) expression as well as pro-inflammatory cytokine secretion such as IL-12, TNF α and IL-6. Altogether, although mDCs are able to prime naive T cells and trigger the adaptive immune response, tolerogenic DCs characterised by an immature or a semi-mature phenotype (low expression of CD86, CD40) or by an altered cytokine secretion profile (low IL-12 and high IL10) are known to induce regulatory T lymphocytes responsible for antigen-specific tolerance. In order to evaluate the stress/activation induced by the micelles, DC maturation was investigated by following the relative expression levels of MHC class II and co-stimulatory molecules by FACS analyses (Fig. 7) and by quantification of the IL-12, TNF α and IL-6 secretion by ELISA (Fig. 8). In these experiments, as a positive control, the full DC maturation was obtained after incubation with 1 μ g/mL of LPS during 24 hours. We clearly observed that DC maturation was dependent on the micelle concentration with a weak up-regulation of CD40 and CD86 molecule expression with the micelles, concentration corresponding of 0.013 mg/mL of copolymer. A significant higher expression of these molecules was observed with the stock solution of the micelles (concentration corresponding of 0.13 mg/mL of copolymer). The DC maturation induced by the addition of the micelles was not complete compared with the LPS-induced maturation. These results were confirmed by the quantification of the pro-inflammatory cytokines. Indeed, the concentrations of TNF α and IL-6 in the supernatant of DCs co-cultured with the diluted solution of the micelles (249 pg/mL and 471 pg/mL respectively) were similar to the levels obtained with control iDCs (259 pg/mL and 138 pg/mL respectively). The stock solution of the micelles incubated with the DCs induced an increase of the TNF α and IL-6 secretion (725 pg/mL

and 1845 pg/mL respectively). The levels of pro-inflammatory cytokines induced by the co-culture of DCs with the stock solution of the micelles were lower than the ones obtained with the fully mDCs (1300 pg/mL for TNF α and 8250 pg/mL for IL-6). In our experiments no IL-12 secretion was detected in the supernatant of micelles co-cultured with DCs, confirming the incomplete maturation process induced by the addition of the micelles (data not shown). The maturation induced by the addition of the micelle stock solution was unlikely due to endotoxin contaminations of the preparations, as the levels of endotoxins of the solutions of each compound and of the formulated micelles were less than 0.25 UI/mL. Thus, in our study the maturation induced by the polyion complex micelles seems to be dose-dependent. A similar result was previously described using poly(lactic-co-glycolic acid) endotoxin-free microparticles on monocytes derived DCs [28]. The maturation process induced by the studied micelles needs further analyses to understand the mechanisms responsible for the maturation induced by non viral particles. The control of the DC maturation depending on the concentration of the polyion complex micelles is really important since the majority of the reported DC-specific vectors such as viral vectors [29], liposomes [30], poly(D-L lactic –co-glycolic) acid nanospheres [31] or microspheres [32] induce DC maturation. All these vectors, as well as high concentrated PMAA₂₁₀₀-b-POE₅₀₀₀/PLL micelles could be used to design cancer vaccines as they imply DC maturation to be able to prime naive T cells. However the control of DC maturation is an important challenge in immunotherapy and the formulated PMAA₂₁₀₀-b-POE₅₀₀₀ micelles used at low concentration seem to be a new powerful tool to restore antigen-specific tolerance by immunomodulatory DCs [33]. Such therapeutical strategy has been reinforced by recent data showing that polymeric particle-engineered dendritic cells were able to induce regulatory T cells [34].

4. Conclusion

DHBC-based polyion complex micelles were prepared using polymethacrylic acid -b- polyethylene oxide and poly-L-lysine and characterised for future cell therapy using dendritic cells. These micelles were stable in physiological conditions (pH and ionic strength), due to a specific core organisation, and disassemble in acidic medium. The cytotoxicity and endocytosis of such micelles were evaluated on murine bone marrow-derived dendritic cells, the vector of choice to use as a cell-based vaccine in immunotherapy. Importantly the good biocompatibility of such vectors, permitting the control of the DC

maturation, widens their application to cell-based immunomodulation in autoimmune diseases in order to restore tolerance.

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Fig.1: Principle of PMAA₂₁₀₀-POE₅₀₀₀/PLL micelle formation (A) and theoretical stability zone as a function of ionisation degree of the two compounds (B) corresponding to the compound pK respectively, 5.5 and 10.5.

Fig. 2: Scattered intensity (full circles) zeta potential (dotted circles) and alpha helix percentage (open circles) as a function of molar ratio of [NH₂]/[COOH] (A) and fluorescent polarization of FITC labelled-poly-L-lysine in presence of increasing amounts of copolymer (B) at pH 7.4.

Fig 3: Circular dichroism analysis of micelles and of free poly-L-lysine in solution (inset graph) as a function of final pH (A) and I1/I3 ratio of pyrene spectra depending on the pH of a solution of PMAA₂₁₀₀-POE₅₀₀₀ and poly-L-lysine at a molar ratio of 1 (B).

Fig. 4: Hydrodynamic diameter and scattered intensity measured by DLS as a function of pH (A) and as a function of NaCl concentration (B) of the final micelle solutions at a molar ratio of 1.

Fig. 5: Follow-up of endocytosis kinetic of micelles fabricated with FITC-PLL.

Fig 6: Fluorescence microscopy of iDC after 3h incubation of FITC-PLL fabricated micelles and DAPI at 37 °C (A and B) and at 4°C (C and D). Overlay of DAPI and FITC signals (A and C) and FITC signal alone (B and D), scale bar = 10µm.

Fig. 7: Phenotypical changes of iDC following 24 h incubation with micelles.

Fig. 8: TNF α and IL-6 quantification by ELISA after 24 h incubation with micelles.

Table 1: Alpha helix percentage as a function of pH of free PLL in solution and PLL fabricated in micelles.

Figure 1
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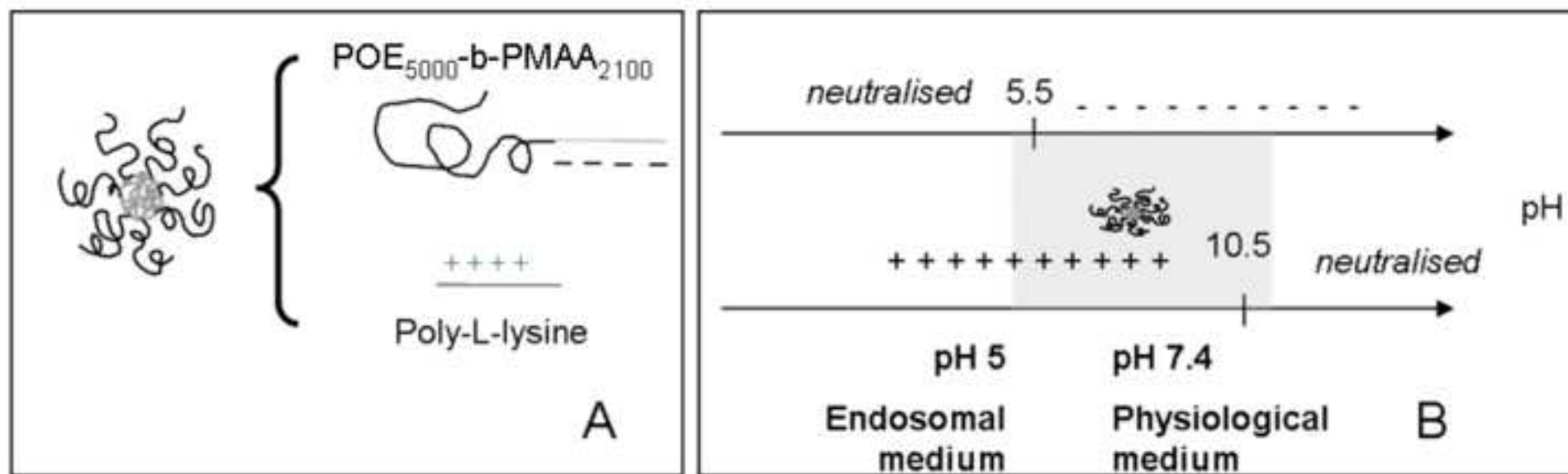


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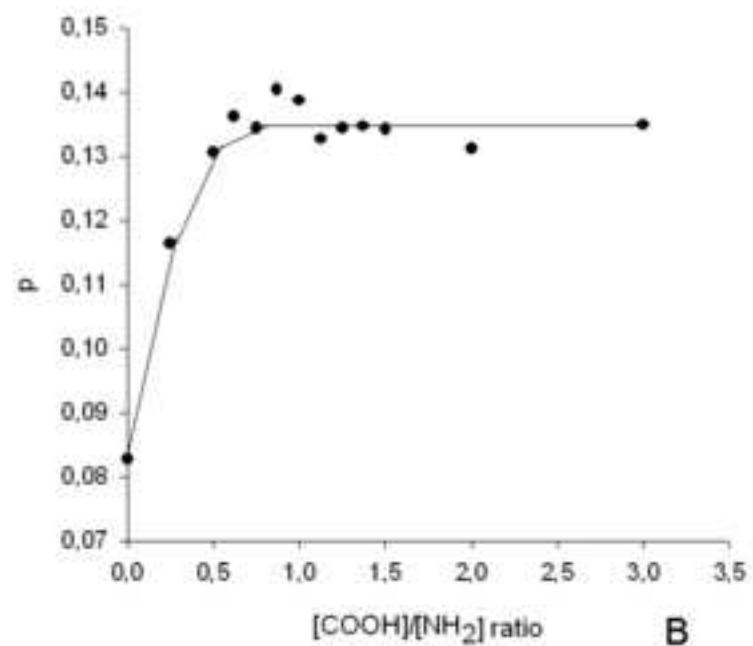
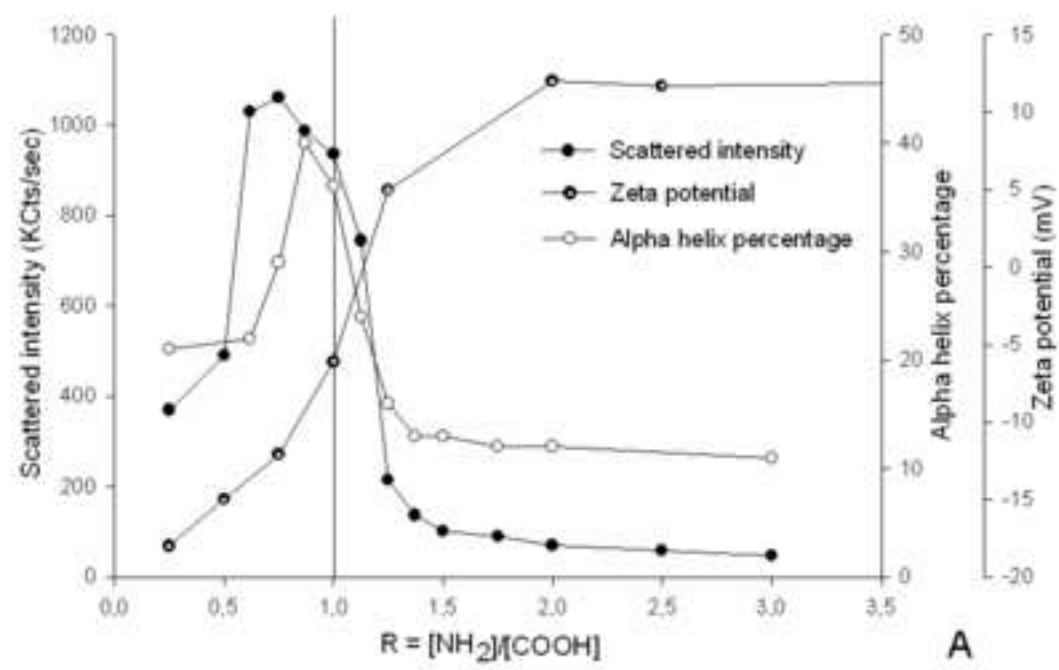


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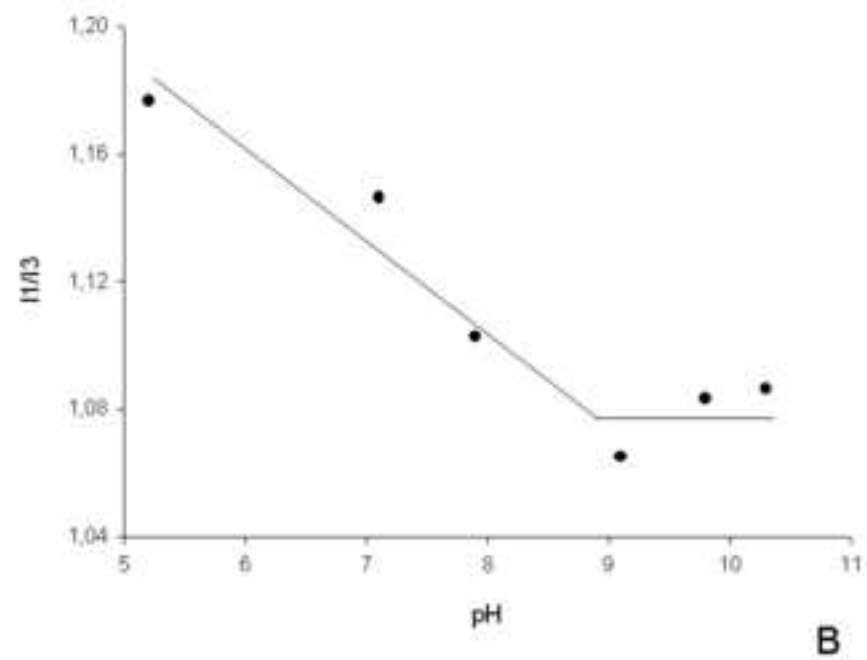
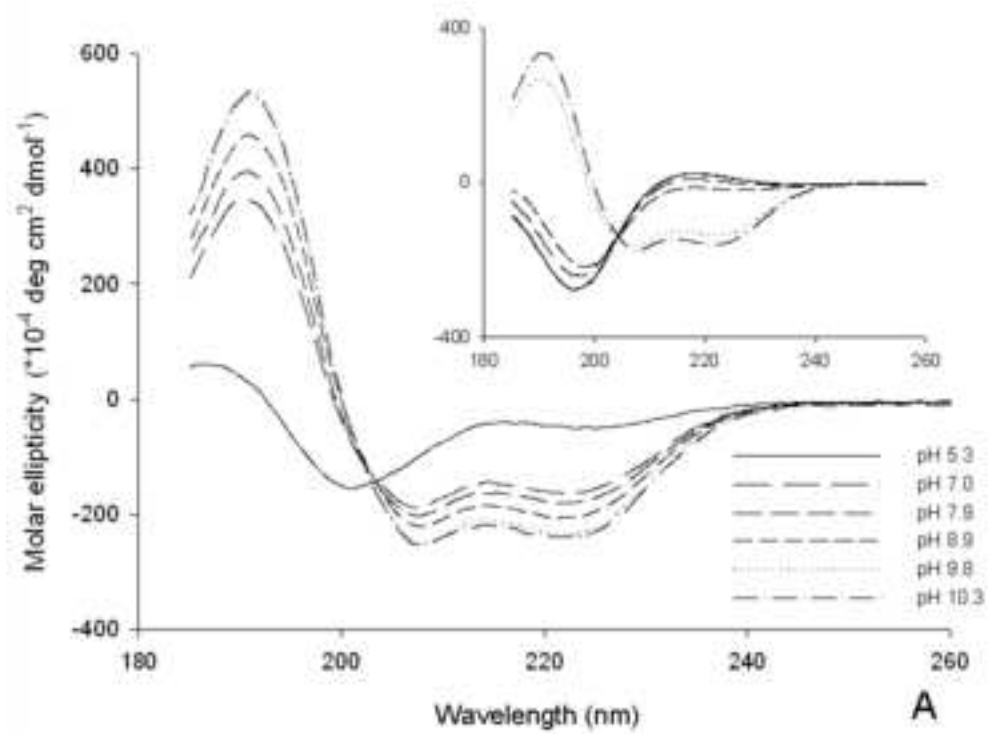


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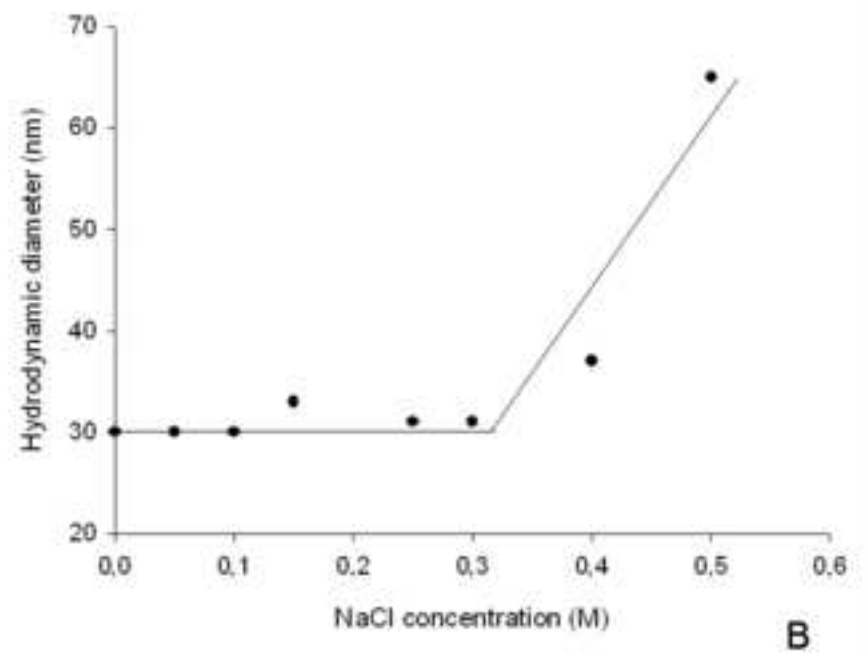
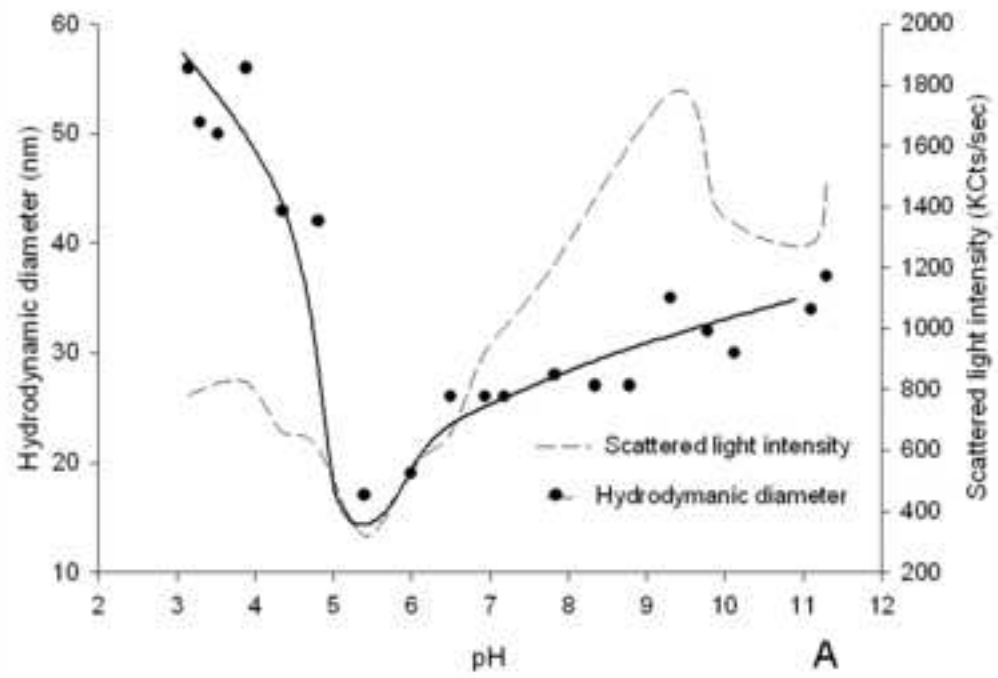


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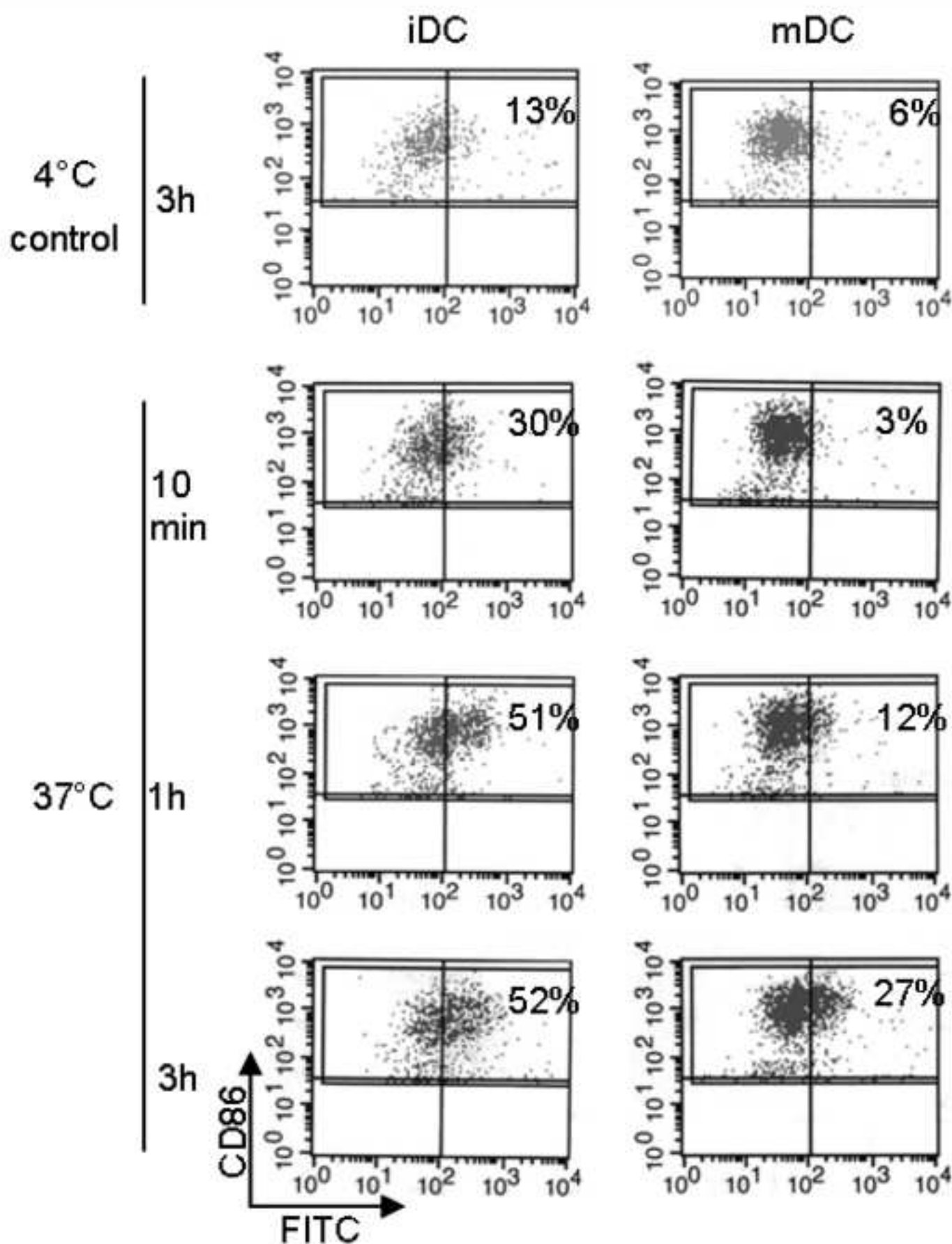


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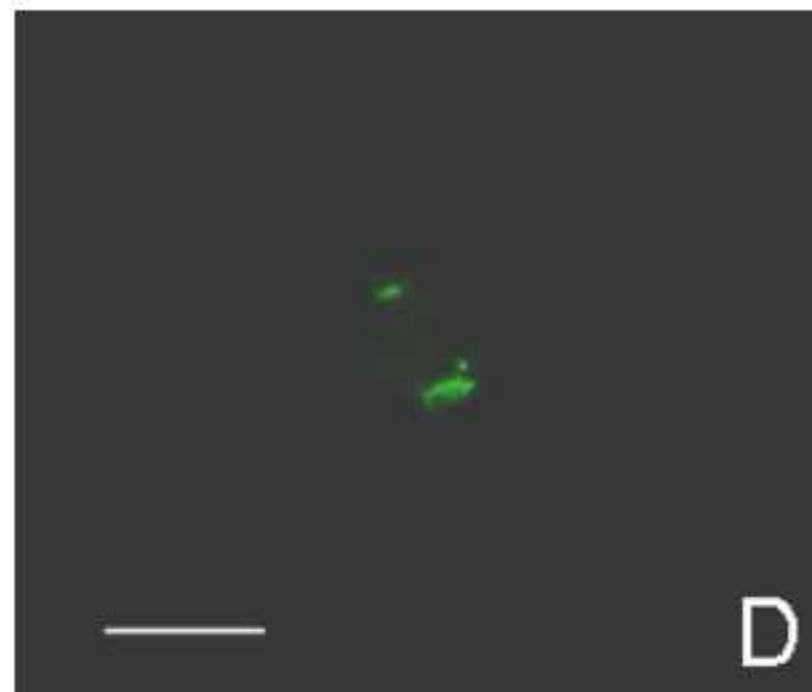
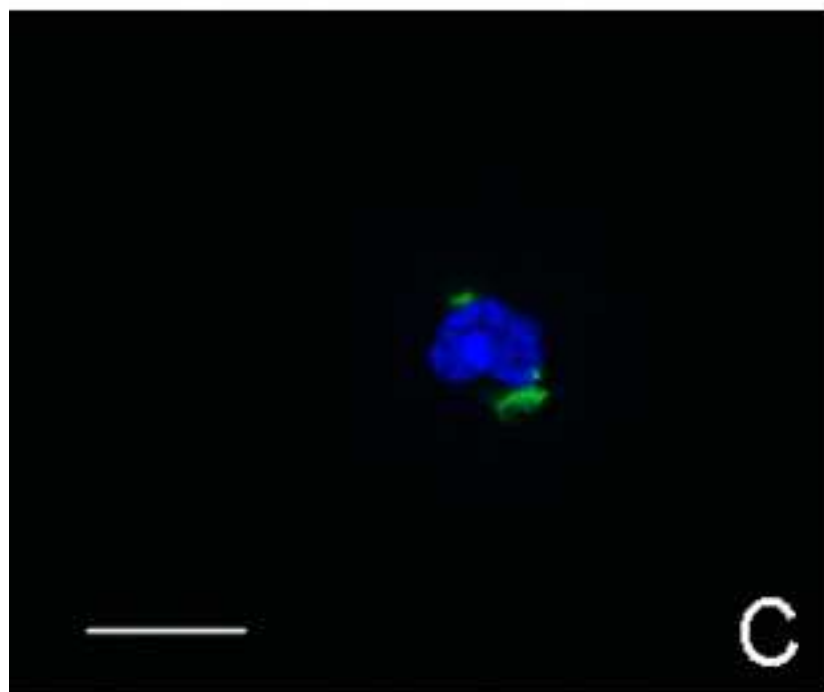
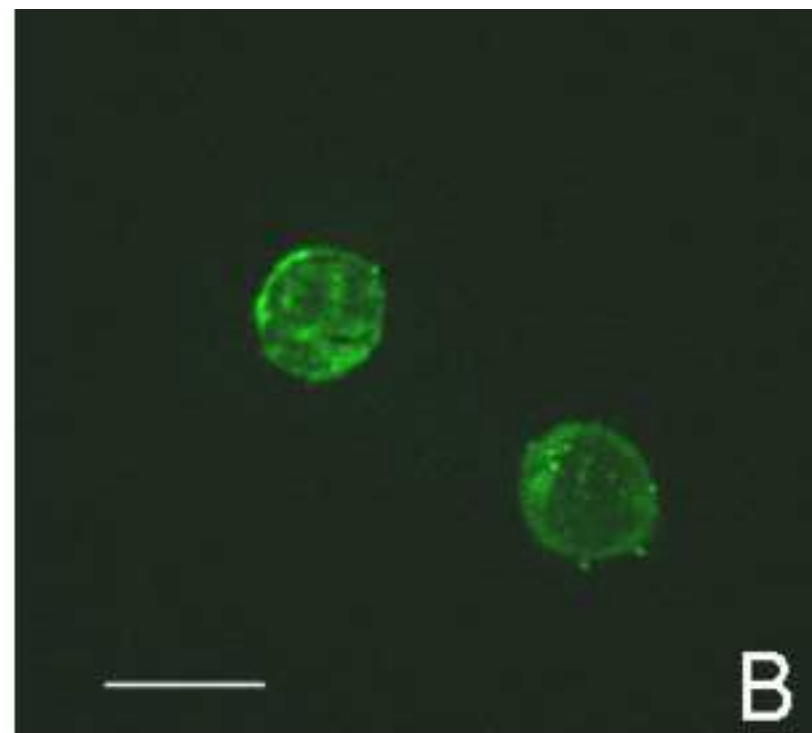
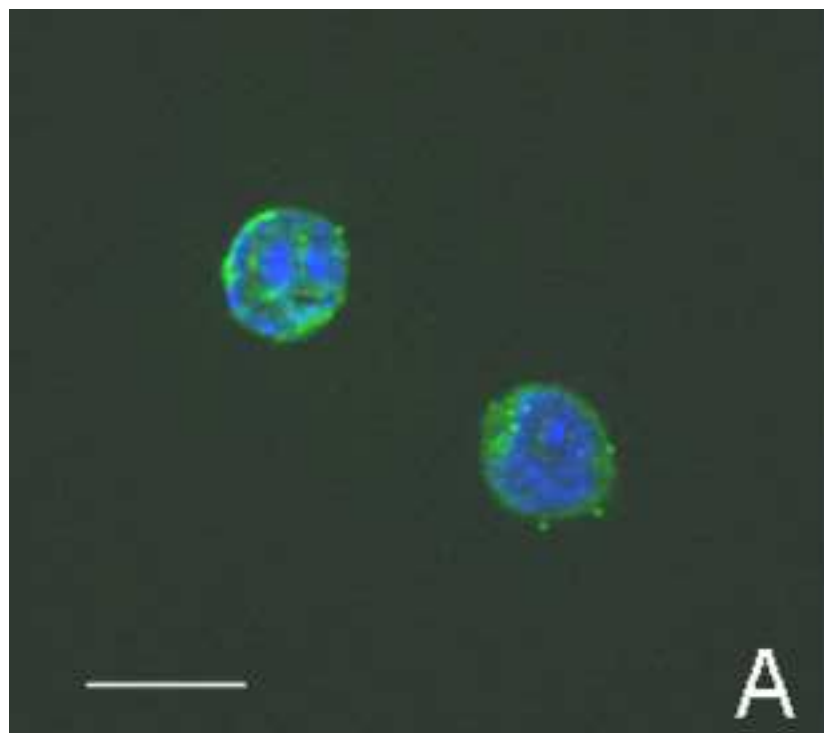


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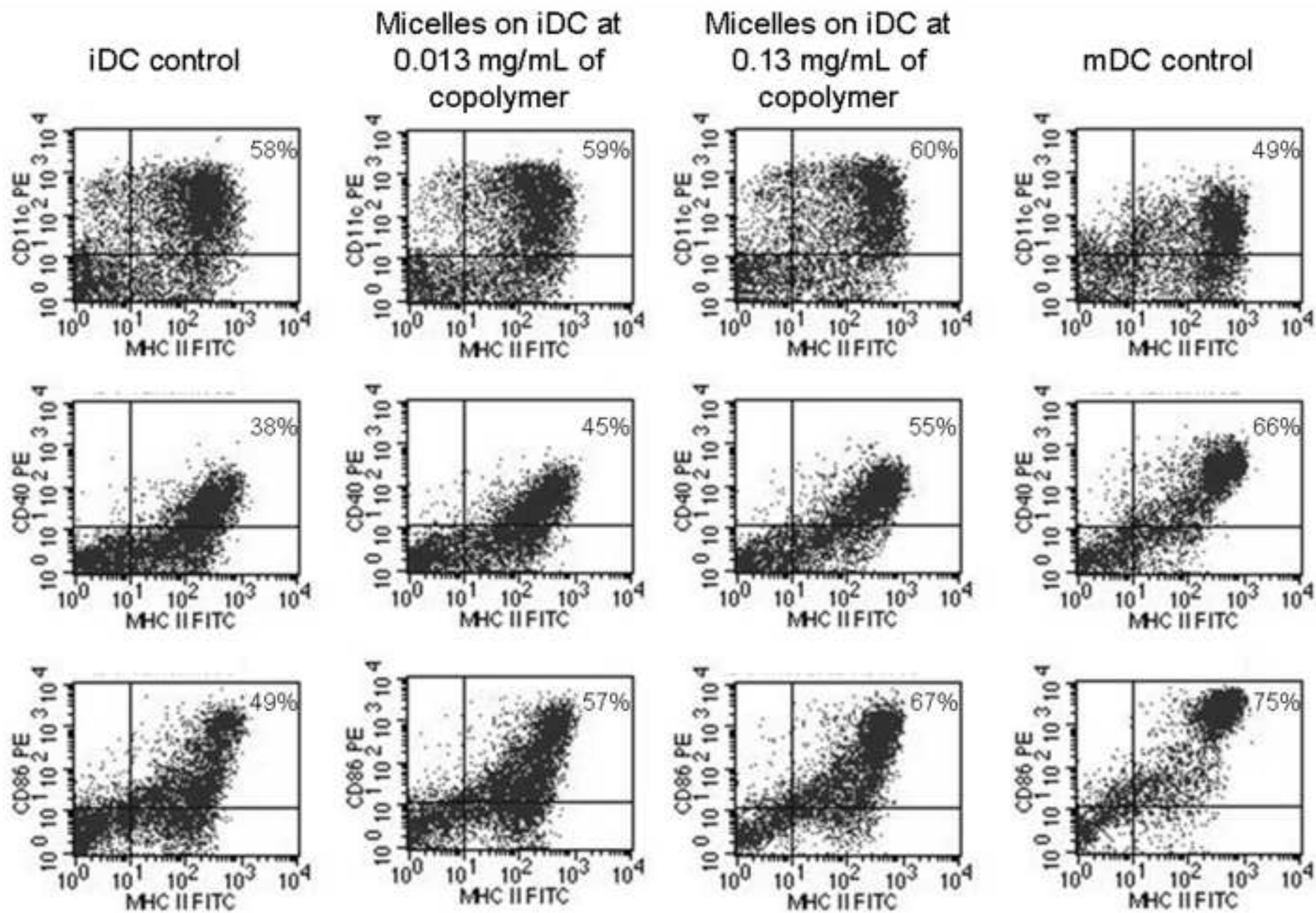


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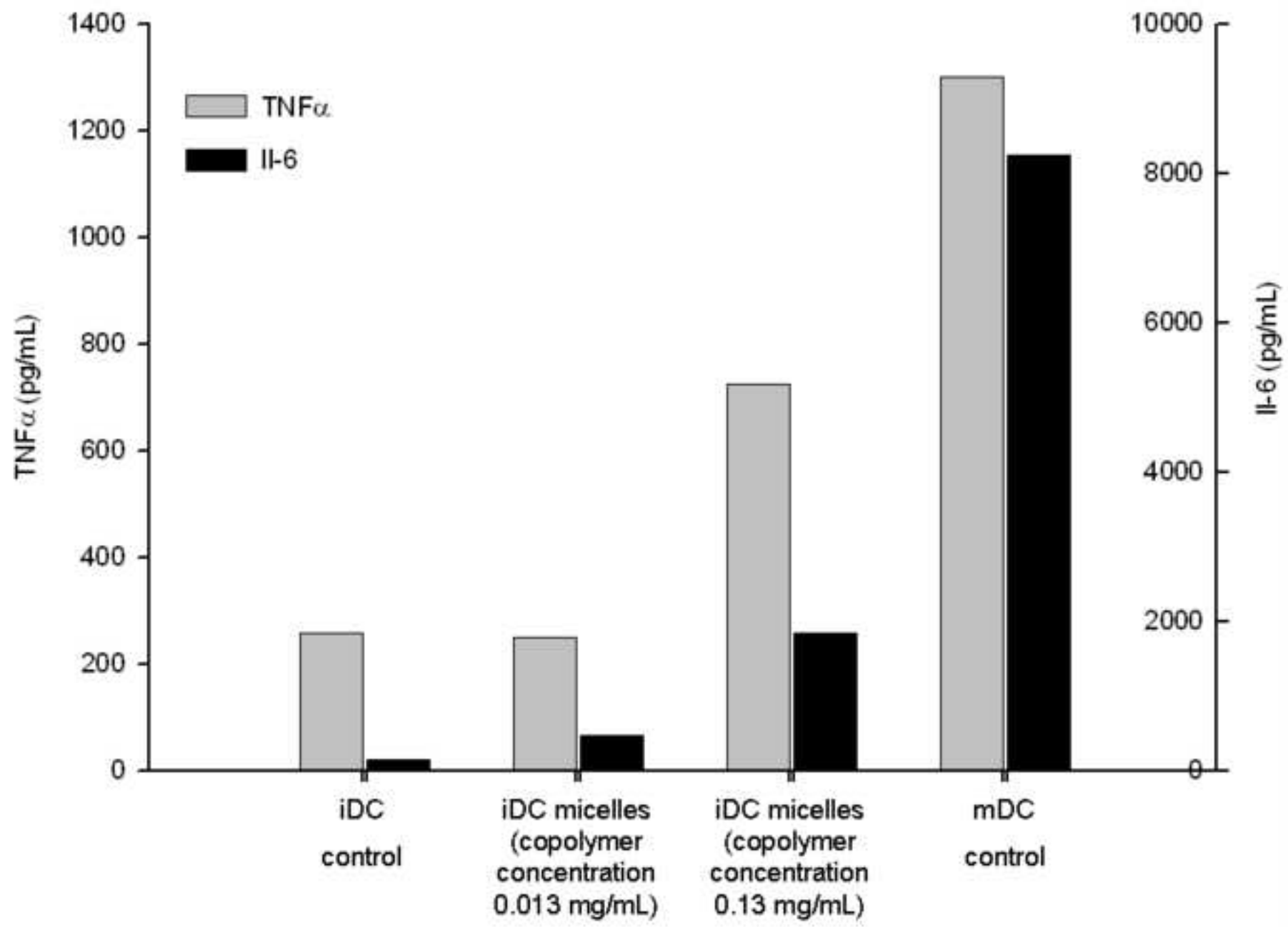


Table 1

pH	Poly-L-lysine in solution	Poly-L-lysine in micelles
5.3	5 %	18 %
7.0	10 %	49 %
7.9	11 %	56 %
8.9	13 %	64 %
9.8	41 %	71 %
10.3	50 %	78 %

Table 1