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Research Paper

Versatile polyion complex micelles for peptide and siRNA vectorization to engineer tolerogenic dendritic cells

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ABSTRACT

Dendritic cells (DCs) are professional antigen-presenting cells that play a critical role in maintaining the balance between immunity and tolerance and, as such are a promising immunotherapy tool to induce immunity or to restore tolerance. The main challenge to harness the tolerogenic properties of DCs is to preserve their immature phenotype. We recently developed polyion complex micelles, formulated with double hydrophilic block copolymers of poly(methacrylic acid) and poly(ethylene oxide) blocks and able to entrap therapeutic molecules, which did not induce DC maturation. In the current study, the intrinsic destabilizing membrane properties of the polymers were used to optimize endosomal escape property of the micelles in order to propose various strategies to restore tolerance. On the first hand, we showed that high molecular weight (Mw) copolymer-based micelles were efficient to favor the release of the micelle-entrapped peptide into the endosomes, and thus to improve peptide presentation by immature (i) DCs. On the second hand, we put in evidence that low Mw copolymer-based micelles were able to favor the cytosolic release of micelle-entrapped small interfering RNAs, dampening the DCs immunogenicity. Therefore, we demonstrate the versatile use of polyionic complex micelles to preserve tolerogenic properties of DCs. Altogether, our results underscored the potential of such micelle-loaded iDCs as a therapeutic tool to restore tolerance in autoimmune diseases.

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1. Introduction

Dendritic cells (DCs) are the most potent antigen presenting cells (APCs) of the immune system, able to both initiate and shape the adaptive immune response triggering both the strength and the quality of the T cell response [1]. At steady state, immature (i) DCs patrol the body to capture antigens, including self-antigens, pathogens and malignant cells. These antigen-loaded DCs migrate to the secondary lymphoid organs and the internalized antigen is processed and presented to T lymphocytes along with appropriate co-stimulation to initiate antigen-specific immune responses. Depending on the context in which the antigen is captured, and notably the cytokine environment or the presence of danger signal, DCs can induce tolerance or immunity. Indeed, DCs respond to antigens and molecules containing pathogen- or damage associated molecular patterns that trigger DC maturation leading to an effective immunity. DCs are not only critical for the induction of primary immune responses, but are also important for the induction of immunological tolerance maintaining the immune homeostasis. DC tolerogenic properties are based on their immature phenotype, cytokine secretion profile and weak immunogenicity [2,3]. Hence, several studies showed iDC capacity to mediate T cell tolerance [4] by using different mechanisms including low expression of CD40, CD80, CD86 co-stimulatory molecules (reviewed in [5]), activation and/or induction of regulatory T cells (Treg) [6], and production of immunosuppressive factors such as IL-10 and TGF-β [7,8].

Their broad range of powerful immune stimulatory and regulatory functions has placed DCs as crucial target cells that could be used as therapeutic tools either to boost effective anti-tumor and anti-infectious immune responses or to alleviate autoimmunity or graft rejection. Whereas clinical trials using immunogenic DCs to treat cancers have been developed over the last 15 years [9,10] tolerogenic DC therapy is just emerging in the clinic.

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Recently, the first phase I clinical trial using tolerogenic DC was reported in type 1 diabetes [11] and two clinical trials in rheumatoid arthritis are ongoing [12,13]. These studies underscore the emergence of tolerogenic DC therapy as an interesting and innovative approach to treat autoimmune diseases.

Several strategies can be used to enhance DC tolerogenic properties: increasing peptide-presentation by iDCs, silencing immunogenic molecules or impeding DC maturation [14,15]. Indeed, pharmacological agents such as IL-10 [16], rapamycin [17], vitamin D3 [18] and other drugs (reviewed in [19]) have been used to manipulate DCs, as well as the use of antisense oligonucleotides targeting immunogenic molecules such as CD40, CD80 and CD86 [11]. More recently, small interfering RNAs (siRNAs) have emerged as a powerful technique for sequence-specific gene silencing providing a potent tool to facilitate the study of gene function in immune cells. Viral vectors are the most commonly used vectors to manipulate cells due to their high and stable transgene expression [20]. However, they are considered as a danger signal by DCs, triggering their maturation by endosomal Toll like receptor activation. Whereas this vector-induced maturation could provide a beneficial adjuvant effect to boost the immune response (reviewed by Humbert et al. [21]) such DC maturation is not suitable for therapeutic strategies in autoimmune diseases and transplantation. To overcome this specific drawback of DC engineering using viral vectors, electroporation [22–24], lipid-based transfection [25–28], and microparticles-based vectors [29–38] with different physical properties and ability to entrap various therapeutic molecules have been developed. However the aim of these studies was to strengthen the immune response and to use the microparticle formulations as cellular vaccines for immunotherapy to increase immunogenicity of the vaccination strategy. We recently developed polyionic micelles with a double hydrophilic block copolymer of poly(methacrylic acid)-*b*-poly(ethylene oxide) (PMAA-*b*-PEO) and a counter polyion, poly-L-lysine (PLL). These pH-sensitive micelles are not recognized as a danger signal by the DCs since they did not trigger their maturation at low concentration. They appear thus particularly suitable for tolerogenic DC engineering [39].

In a preliminary study, we demonstrated that antigenic peptide as well as siRNAs could be efficiently entrapped into the $PMAA_{2100}$-*b*-PEO_{5000} polyionic micelles according to an original tripartite association [40,41]. However, to further enhance peptide presentation and to favor endosomal escape of siRNA molecule for an efficient silencing, we recently optimized such PMAA-*b*-PEO polyionic micelles using various PMAA and PEO with intrinsic membrane destabilizing properties [42]. In the present study, taking advantage of these destabilizing properties, we formulated high and low molecular weights (Mw) copolymer-based micelles either with OVA peptide or with siRNA targeting the co-stimulatory CD86 molecule as proof of concept to enhance peptide presentation by iDCs or to dampen immunogenicity of the DCs respectively. The DC uptake of labeled-micelles as well as the micelle ability to deliver the entrapped molecules to their appropriate target sites was quantified using fluorescent-PLL or fluorescent-entrapped molecules respectively. Our results clearly underscored the potential of such versatile and non-immunogenic PMAA-*b*-PEO based-micelles to restore tolerance in autoimmune disease using both strategies.

2. Materials and methods

2.1. Materials

PMAA-*b*-PEO copolymers with low ($PMAA_{2100}$-*b*-PEO_{5000}, $PMAA_{3500}$-*b*-PEO_{5000}) and high Mw ($PMAA_{2500}$-*b*-$PEO_{12,000}$, $PMAA_{16,200}$-*b*-$PEO_{30,000}$) were obtained from Polymer Source Inc. (USA). The PLL hydrobromide ($pK_b = 10.5$, Mw 15,000–30,000 g/mol) and its fluorescein isothiocyanate-conjugated (FITC, λ_{ex}: 419 nm and λ_{em}: 518 nm) were purchased from Sigma Aldrich (France). PLLs conjugated with cyanine 5 (Cy5, λ_{ex}: 683 nm and λ_{em}: 707 nm) and cyanine 3 (Cy3, λ_{ex}: 552 nm and λ_{em}: 570 nm) were supplied by Interchim (France). All the polymers were solubilized in phosphate buffer saline solution (PBS), pH 7.4. The $OVA_{323-339}$ peptide (ISQAVHAAHAEINEAGR, 1772 g/mol) and OVA^{FITC} peptide (2286 g/mol) were synthesized as previously described [40]. OVA^{TAMRA} peptide (2185 g/mol) labeled with tetramethylrhodamine (TAMRA, λ_{ex}: 552 nm and λ_{em}: 575 nm) was purchased from AnaSpec Inc. (USA). siRNA targeting CD86 (5′-GCCUGAGUGAGCUGGUAGUAU-3′) and the non-targeting fluorescent $siRNA^{TAMRA}$ were purchased from CureVac (Tubingen, Germany), and the non-targeting fluorescent control $siRNA^{siGLO}$ (RISC-free control siRNA) was purchased from Dharmacon (ThermoFisher, UK). The transfection agent Lipofectamine® 2000 was supplied by Invitrogen (France).

2.2. Membrane destabilization with PMAA-b-PEO copolymers

The membrane destabilizing capacity of the copolymers was studied as a function of the copolymer concentration using a red blood cell (RBC) hemolysis assay as previously described [42]. The released hemoglobin following the membrane disruption was quantified by the absorbance at 540 nm using a microplate reader (Multiskan FC, Thermo-Scientific, France).

2.3. Micelle preparation and characterization by Dynamic Light Scattering (DLS)

Empty micelles were prepared as previously described [42]. The OVA^{FITC} peptide-entrapped micelles were prepared by dissolving 70 μg of OVA^{FITC} in 500 μl of PLL solutions, with constant agitation at 4 °C. After 1 h, 500 μl of copolymer solutions were added with overnight agitation. Similarly, siRNA-entrapped micelles were prepared by adding siRNA and PLL solutions, with constant agitation at 4 °C for 30 min and subsequent addition of the copolymer solutions. Size distribution and zeta potential of empty micelles, OVA^{FITC} peptide- or siRNA-entrapped micelles were determined by DLS using a Nano®ZS instrument (Malvern Instrument, UK). The autocorrelation function was modeled by using the Non-Negative Least Squares algorithm.

Filtration of the OVA^{FITC}-entrapped and PLL^{Cy5}-formulated micelles was performed using a size-exclusion PD-10 column (GE Healthcare, USA) to completely remove any free OVA^{FITC} or PLL^{Cy5}. Due to the 1/3 dilution, associated with this separation process, the filtrated micelles corresponded to a polymer (PMAA-*b*-PEO + PLL) concentration of 280, 337, 345 or 370 μg/ml for the $PMAA_{2500}$-*b*-$PEO_{12,000}$, $PMAA_{2100}$-*b*-PEO_{5000}, $PMAA_{16,200}$-*b*-$PEO_{30,000}$ and $PMAA_{3500}$-*b*-PEO_{5000} based micelles, respectively. These copolymer concentrations were used to calculate the degree of OVA-entrapment, which corresponds to the ratio of the amount of entrapped OVA peptide on the polymer amount. The quantification of free OVA^{FITC} peptide or micelle-entrapped OVA^{FITC} peptide was carried out by fluorescence measurements using a RF 5302 Shimadzu spectrofluorometer (Japan) equipped with a xenon light source (UXL-150S, Ushio, Japan). The calibration curve was done using OVA^{FITC} peptide solutions in PBS and displayed a good linearity and sensitivity ($r^2 = 0.99$, 0.1–0.8 μg/ml). Micelles siRNA-entrapment was measured using micelles formulated with 2.5% of PLL^{FITC} and increasing concentrations of non-targeting fluorescent $siRNA^{TAMRA}$ by Fluorescence Resonance Energy Transfer (FRET). Sample spectra were recorded using a RF 5302 Shimadzu spectrofluorometer. The siRNA-entrapment efficacy of PMAA-*b*-

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