Acta Biomaterialia 10 (2014) 1847–1855

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

# Macrophage mannose receptor-specific gene delivery vehicle for macrophage engineering

Gui-Xin Ruan [a], Yu-Zhe Chen [a], Xing-Lei Yao [a], Anariwa Du [b], Gu-Ping Tang [c], You-Qing Shen [d], Yasuhiko Tabata [e], Jian-Qing Gao [a,*]

[a] Zhejiang Province Key Laboratory of Anti-Cancer Research, College of Pharmaceutical Sciences, Zhejiang University, 866 Yuhangtang Road, Hangzhou 310058, Zhejiang, People's Republic of China
[b] Department of Clinical Laboratory, Inner Mongolia Peoples Hospital, 20 Zhaowuda Road, Huhehaote 010010, Inner Mongolia, People's Republic of China
[c] Institute of Chemical Biology and Pharmaceutical Chemistry, Zhejiang University, Hangzhou, People's Republic of China
[d] Center for Bionanoengineering and State Key Laboratory of Chemical Engineering, Zhejiang University, Hangzhou 310027, People's Republic of China
[e] Department of Biomaterials, Field of Tissue Engineering, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan

ARTICLE INFO

*Article history:*
Received 12 October 2013
Received in revised form 15 December 2013
Accepted 9 January 2014
Available online 16 January 2014

*Keywords:*
Gene delivery
Mannose receptor
Macrophage
Spermine–mannan

ABSTRACT

Macrophages are the most plastic cells in the hematopoietic system and they exhibit great functional diversity. They have been extensively applied in anti-inflammatory, anti-fibrotic and anti-cancer therapies. However, the application of macrophages is limited by the efficiency of their engineering. The macrophage mannose receptor (MMR, CD206), a C-type lectin receptor, is ubiquitously expressed on macrophages and has a high affinity for mannose oligosaccharides. In the present study, we developed a novel non-viral vehicle with specific affinity for MMR. Mannan was cationized with spermine at a grafted ratio of ~12% to deliver DNA and was characterized as a stable system for delivery. This spermine–mannan (SM)-based delivery system was evaluated as a biocompatible vehicle with superior transfection efficiency on murine macrophages, up to 28.5-fold higher than spermine–pullulan, 11.5-fold higher than polyethylenimine and 3.0-fold higher than Lipofectamine™ 2000. We confirmed that the SM-based delivery system for macrophages transfection was MMR-specific and we described the intracellular transport of the delivery system. To our knowledge, this is the first study using SM to demonstrate a mannose receptor-specific gene delivery system, thereby highlighting the potential of a novel specific non-viral delivery vehicle for macrophage engineering.


## 1. Introduction

Macrophages are the most plastic cells in the hematopoietic system and show great functional diversity. They participate in almost every aspect of organism biology, from development, homeostasis and repair to immune responses to pathogens [1]. Macrophages are functionally divided according to a binary classification based on inflammatory state: classically activated macrophages (M1) and alternatively activated macrophages (M2), which respectively correspond to the Th1/Th2 programming of adaptive immune cells [1]. Macrophages also play important roles in many chronic diseases, including rheumatoid arthritis and fibrosis, and they are thought to promote key processes in tumor progression [1,2]. These functions have prompted recent attempts to utilize macrophages as novel cellular vehicles for therapy, wherein macrophages are genetically modified in vitro or are directly designed to target macrophages in vivo [3]. However, achieving sufficient gene expression in macrophages in vitro or manipulating it through macrophage-specific transfection in vivo has proven problematic. Thus, macrophage-specific or targeted gene delivery systems seem to be a prospective approach to improving therapeutic efficacy.

Gene delivery systems typically consist of physical and chemical methods, viral delivery systems and non-viral delivery systems (the so-called synthetic vehicles). Aside from these methods, several studies have also exploited microorganisms (bacteria or protozoan) as vehicles for delivering therapeutic DNA to macrophages [4,5], but they were relatively ineffective [5], with unknown risks. Physical or chemical methods such as electroporation have also been reported in early studies [6,7], but are limited by their insufficiency and their resulting high levels of cell death – their main disadvantage. Viral delivery systems such as adenoviruses, retroviruses, lentiviruses and adeno-associated viruses are popular for engineering primary macrophages because of their ability to transduce macrophages with relatively high efficiency [8,9]; however, adenoviruses are reportedly less efficient in macrophage cell line transfection [10]. Immunogenicity and

* Corresponding author. Tel./fax: +86 571 88208437.
*E-mail address:* gaojianqing@zju.edu.cn (J.-Q. Gao).

1742-7061/$ - see front matter © 2014 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.actbio.2014.01.012

mutagenicity are also limitations associated with many recombinant viruses. Moreover, macrophages are quite responsive to danger signals such as those from original viral vehicles, which may induce potent antiviral responses, making these viral delivery systems unsuitable for macrophage engineering [9]. Recent studies have focused on non-viral delivery systems for macrophage engineering because of their ease of administration, larger capacity and lower risk of mutative and immunogenic induction; however, they are limited by their inefficient transfection and lack of specificity. In some early studies, receptor-specific non-viral gene delivery systems have demonstrated relatively high transfection efficiencies on primary macrophages in vitro, although the mechanism has not been elucidated [11,12].

Macrophages have a specific affinity for carbohydrate-based delivery systems via C-type lectin receptors (CLRs); thus, CLRs have been the focus of delivery design. CLRs are carbohydrate-binding molecules that bind ligands in a $Ca^{2+}$-dependent manner and are ubiquitously expressed on antigen-presenting cells, such as DC-SIGN (CD209) and DEC-205 (CD205) expressed on dendritic cells (DCs), langerin (CD207) on Langerhans cells and macrophage mannose receptor (MMR, CD206) on macrophages and DCs [13,14]. MMR, which belongs to the mannose receptor (MR) family, is a type I transmembrane receptor with three types of extracellular domains: a functional cysteine-rich (CR) domain, eight C-type lectin-like domains (CTLD) and a fibronectin domain [15,16]. Among MRs, only MMR and Endo-180 bind carbohydrates in a $Ca^{2+}$-dependent manner [13]. MMR binds mannose-bearing structures and enhances the uptake of mannosylated antigens, which has potential applications in the design of macrophage-specific delivery systems. After detailed characterization of receptor expression on macrophages, several reports have focused on designing non-viral delivery systems specific for the mannose receptors on macrophages and DCs [17–19], particularly the mannose-mediated delivery system [20–22]. Most of these designs are based on the mannosylated modification of gene delivery materials.

Instead of mannosylation, we selected a mannan backbone, which has an affinity for mannose receptors, to develop a cationic polymer. Mannan from *Saccharomyces cerevisiae* possesses a backbone of α-(1-6)-linked D-mannose units, which are substituted by side chains containing (1-2)- and (1-3)-linked D-mannose units [23]. Thus, mannan has a higher affinity for mannose receptors than monosaccharides. To deliver negatively charged DNA or RNA, the mannan backbone is conjugated with spermine, a naturally occurring tetramine that displays superior properties compared to other amine types [24]. The resulting spermine–mannan (SM) system was characterized, evaluated using different cell lines to determine the optimal N/P ratio for transfection and compared with other classical non-viral vehicles. The mechanism by which the SM-based delivery system was internalized and transported intracellularly was also investigated.

## 2. Materials and methods

### 2.1. Reagents

Mannan from *S. cerevisiae* (average molecular weight of 100 kDa), 1,1′-carbonyl-diimidazole (CDI) and spermine were purchased from Sigma–Aldrich (St Louis, MO, USA). The pGL3 control was purchased from Promega, and fluorescein isothiocyanate (FITC)-DNA was synthesized by Sangon Biotech Co. Ltd (Shanghai, China), using a sequence of 50 thymine residues and FITC modification.

### 2.2. Cell lines

HeLa, B16BL6 and RAW264.7 cells were purchased from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China. DC2.4 cells were provided by the Department of Immunology, Zhejiang University, Hangzhou, China. All cell lines were cultured at 37 °C with 5% $CO_2$. The HeLa and RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Boster, Wuhan, China) containing 10 vol.% fetal bovine serum (FBS; Hyclone, Utah, USA). The B16BL6 cells were cultured in RPMI-1640 containing 10 vol.% bovine calf serum (BCS; Gibco, Carlsbad, CA, USA). The DC2.4 cells were cultured in RPMI-1640 (Boster, Wuhan, China) containing 10 vol.% FBS.

### 2.3. Synthesis and characterization of SM

Exactly 25 mg of mannan (20–200 kDa) was dissolved in 10 ml of anhydrous dimethyl sulfoxide (DMSO) and stirred to complete dissolution at 60 °C. Also, 218 mg of CDI (a 9:1 M ratio to the $-CH_2-OH$ content of mannan) was dissolved in DMSO and gradually added to the dissolved mannan solution to activate the $-CH_2-OH$ at 40 °C for 30 min. The activated mannan solution was added dropwise to spermine solution (at a molar ratio of 3:1 to CDI) in 20 ml DMSO, and allowed to react for 18 h at 37 °C. The mixture was dialyzed for 48 h using 8000–14,000 MW cutoff membrane in distilled deionized water and the product was collected for lyophilization. Solid SM was prepared for elemental analysis and infrared (IR) spectrophotometric analysis, and was further prepared in d-$H_2O$ for proton nuclear magnetic resonance ($H^1$-NMR) analysis.

### 2.4. Preparation and characterization of the SM/DNA complex

SM was dissolved in PBS and merged with pGL3-control or FITC-DNA (100 μg $ml^{-1}$) in an equal volume of PBS, at different molar ratios of the nitrogen in the SM and the phosphorus in the DNA (abbreviated as the N/P ratio). The mixture was maintained at room temperature for more than 15 min. The particle size and zeta potential were measured using a Malvern Zetasizer (Zetasizer 3000 HSa, Malvern). The morphology was observed under a transmission electron microscope (TEM, H-9500, Hitachi). The stability of the conjugated complex was determined at different N/P ratios using DNA agarose gel electrophoresis (AGE), as previously described [25].

### 2.5. Transfection evaluation

HeLa and B16BL6 cells were cultured at $5 \times 10^4$ cells per well and the DC2.4 and RAW264.7 cells were cultured at $10^5$ cells per well in 24-well plates for 24 h. SM/DNA complexes were prepared at N/P ratios of 1, 3, 5, 7, 10 and 20. Spermine–pullulan (SP)/DNA complexes were prepared at an N/P ratio of 3 [26]. Polyethylenimine (branched 25 kDa PEI, Sigma)/DNA complexes were prepared at an N/P ratio of 10 [27–29]. Lipofectamine™ 2000 (Lipo, Invitrogen, Carlsbad, CA, USA)/DNA complexes were prepared following the manufacturer's instructions. The complexes were added to cells with plasmid mass of 1 μg per well, and incubated in serum-free medium for 6 h. The medium was replaced with complete medium and cultured for another 18 h. The luciferase expression levels were quantified using a luciferase reporter gene assay kit (Beyotime, Shanghai, China) following the administration guidelines. The relative light unit (RLU) was determined using a luminometer (GloMax Jr Multi-Detection System, Promega). Protein concentration was determined using a BCA protein assay kit (Beyotime, Shanghai, China). Luciferase activity is reported as RLU/protein concentration.

### 2.6. Cytotoxicity evaluation

HeLa, B16BL6, DC2.4 and RAW264.7 cells were cultured at $10^4$ cells per well in 96-well plates for 24 h. Vectors/DNA complexes