Biomaterials 35 (2014) 2987–2998

Contents lists available at ScienceDirect

Biomaterials

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

Bacterial imaging with photostable upconversion fluorescent nanoparticles

Li Ching Ong [a], Lei Yin Ang [b], Sylvie Alonso [a,b,**], Yong Zhang [a,c,*]

[a] Graduate School for Integrative Sciences and Engineering, National University of Singapore, Centre for Life Sciences (CeLS), #05-01, 28 Medical Drive, Singapore 117456, Singapore
[b] Department of Microbiology, Immunology Program, National University of Singapore, Centre for Life Sciences (CeLS), #03-05, 28 Medical Drive, Singapore 117456, Singapore
[c] Department of Biomedical Engineering, Faculty of Engineering, National University of Singapore, Block EA #03-12, 9 Engineering Drive 1, Singapore 117575, Singapore

ARTICLE INFO

Article history:
Received 18 November 2013
Accepted 19 December 2013
Available online 10 January 2014

Keywords:
Bacteria labeling
Upconversion nanoparticles
Fluorescence imaging
Escherichia coli

ABSTRACT

Autofluorescence, photodamage and photobleaching are often encountered when using downconverting fluorophores and fluorescent proteins for bacteria labeling. These caveats represent a serious limitation when trying to map bacteria dissemination for prolonged periods. Upconversion nanoparticles (UCNs), which are able to convert low energy near-infrared (NIR) excitation light into higher energy visible or NIR light, can address these limitations. These particles' unique optical properties translate into attractive advantages of minimal autofluorescence, reduced photodamage, deeper tissue penetration and prolonged photostability. Here, we report a UCN-based bacteria labeling strategy using *Escherichia coli* as prototypic bacteria. A comparative analysis highlighted the superior photostability of UCN-labeled bacteria over green fluorescent protein-expressing bacteria. Infection study of UCN-labeled bacteria in dendritic cells indicated co-localization of the UCN signal with bacterial position for up to 6 h post-infection. Furthermore, long-term monitoring of the same infected cells demonstrated the potential to utilize photostable UCN-based imaging for bacterial trafficking purposes.

1. Introduction

The ability for a microbial pathogen to disseminate from its point of entry to other target organs into its host represents a critical aspect of pathogenesis. Importance of dissemination has been illustrated in several disease examples, where the extent of pathogen dissemination to draining lymph nodes or other organs has been directly correlated with disease severity and manifestations [1,2]. Therefore, mapping the trafficking profile of a pathogen is of great interest to gain further insights into host–pathogen interactions and the mechanisms involved in a pathogen's ability to cause disease [3]. Furthermore, for attenuated bacteria and other non-pathogenic bacteria which may be used as vaccine candidates or carriers, information of their biodistribution, persistence and clearance in the host is also essential for establishing their safety and efficacy profiles, and for a rational design of bacteria-based vaccine delivery systems [4].

Current approaches of bacteria labeling generally involve either tagging the bacteria with custom designed fluorescent probes [5–8] or generating recombinant bacteria that express luciferase or fluorescent proteins [3]. Luciferase-expressing recombinant bacteria require the constant presence of its substrate that may be toxic at high concentrations. Furthermore, signal production is highly dependent on bacterial metabolic state and oxygen content in the environment [3,9] On the other hand, when using organic dyes as probes or recombinant bacteria expressing fluorescent proteins, photobleaching, photodamage and autofluorescence issues are commonly encountered. Despite efforts to improve photostability of fluorescent dyes and fluorescent proteins by limiting power exposure [10] and reducing exposure to quenchers [11,12], the problems are not completely resolved, and these approaches still do not allow the study of bacterial dissemination *in vivo* which requires long and/or repeated exposure times. There is thus a need for alternative bacterial labels that can overcome the current limitations.

* Corresponding author. Department of Biomedical Engineering, Faculty of Engineering, National University of Singapore, Block EA #03-12, 9 Engineering Drive 1, Singapore 117575, Singapore. Tel.: +65 6516 4871; fax: +65 6872 3069.
** Corresponding author. Department of Microbiology, Yong Loo Lin School of Medicine, Immunology Program, Life Science Institute, National University of Singapore, Centre for Life Sciences (CeLS) building, #03-05, 28 Medical Drive, Singapore 117456, Singapore. Tel.: +65 6516 3673; fax: +65 6778 2684.
E-mail addresses: micas@nus.edu.sg (S. Alonso), biezy@nus.edu.sg (Y. Zhang).

0142-9612/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2013.12.060

Though efforts have been made to utilize semiconductor-based quantum dots (QDs) as alternative bacterial labels [13–15], the potential cytoxicity risk associated with their heavy metal components such as cadmium remains a major concern for the use of QDs in biological studies [16]. In addition, since the fluorescence from QDs relies on energy downconversion, background autofluorescence remains a concern as shorter wavelength light is used to excite the nanoparticles. Autofluorescence interferes with signal to background ratio and adversely affects detection limits. This has presented a major hurdle for monitoring bacterial trafficking *in vivo* as usually low number of bacteria are tracked. Upconversion nanoparticles (UCNs) represent an emerging class of luminescent nanomaterial, which have been shown to be free of autofluorescence *in vivo* and with a superior detection limit when compared to QDs [17]. These advantages are conferred by the unique ability of UCNs to upconvert light by sequential multiphoton light absorption, where lower energy near-infrared (NIR) excitation light is converted into emission light of higher energy [18–20] The near-infrared excitation wavelength of UCNs lies close to the optical window, a spectral region from 650 nm to 950 nm in which biological materials have low absorption and scattering coefficients, thus leading to negligible autofluorescence and increased tissue penetration [21]. Hence, UCN-based labeling technology has become appealing as it allows for deeper tissue penetration, low autofluorescence and minimal photodamage (as depicted in Fig. 1). In addition, the extreme photostability of these nanoparticles has also been well-established [19]. This implies that monitoring of UCN-labeled entities such as mammalian cells or bacteria for example can be carried out without any decrease of the signal intensity over long periods of time. Last but not least, UCNs have been associated with low cytotoxicity in various *in vitro* and *in vivo* settings, supporting their suitability for biological applications [18,19]. As such, UCN labeling has been successfully reported for various mammalian systems both *in vitro* and *in vivo* [18–20,22]. However, the use of UCNs for bacteria labeling has never been reported before. The successes of labeling and imaging of mammalian cells strongly suggest the feasibility of extending UCN labeling to the bacteria kingdom and help to overcome the limitations currently faced using fluorescent dyes and proteins for bacterial trafficking.

We report here the successful labeling of prototypic *Escherichia coli* (*E. coli*) bacteria with UCNs. The resulting UCN-labeled bacteria exhibited strong UCN luminescence upon NIR excitation. Superior photostability of the UCN-labeled bacteria was demonstrated by comparing its photobleaching profile with that of green fluorescent protein (GFP)-expressing *E. coli*. Monitoring of UCN-labeled bacteria upon dendritic cell infection was also performed and showed good correlation between UCN signal and bacteria positioning.

2. Materials and methods

2.1. Synthesis of antibody-conjugated-UCNs

2.1.1. Synthesis of citrate-UCN

All chemicals used here were purchased from Sigma–Aldrich and used without further purification. Oleic acid-capped $NaYF_4$:Yb,Er UCNs were first synthesized using a previously reported reaction scheme [23]. One mmole of reaction salts (YCl_3, $YbCl_3$ and $ErCl_3$) in the stoichiometric ratio were dissolved in 6 ml of oleic acid and 15 ml of octadecene by heating the mixture at 160 °C for about 30 min until a homogenous solution was formed. After cooling the solution to room temperature, 2.5 mmol of sodium hydroxide and 4 mmol of ammonium fluoride (dispersed in 10 ml of methanol) were added. The mixture was then slowly heated for methanol removal and also degassed at 100 °C for 10 min. After which, the mixture was maintained at 300 °C under an argon atmosphere for 1 h. The nanoparticles were purified by acetone precipitation and the other insoluble impurities were removed by low speed centrifugation. Purified oleic acid-capped-UCNs were stored in cyclohexane until further use for citrate modification.

Citrate modification of the OA-UCNs was performed via a ligand exchange process as described previously [24,25]. Briefly, a mixture of 2 mmol sodium citrate

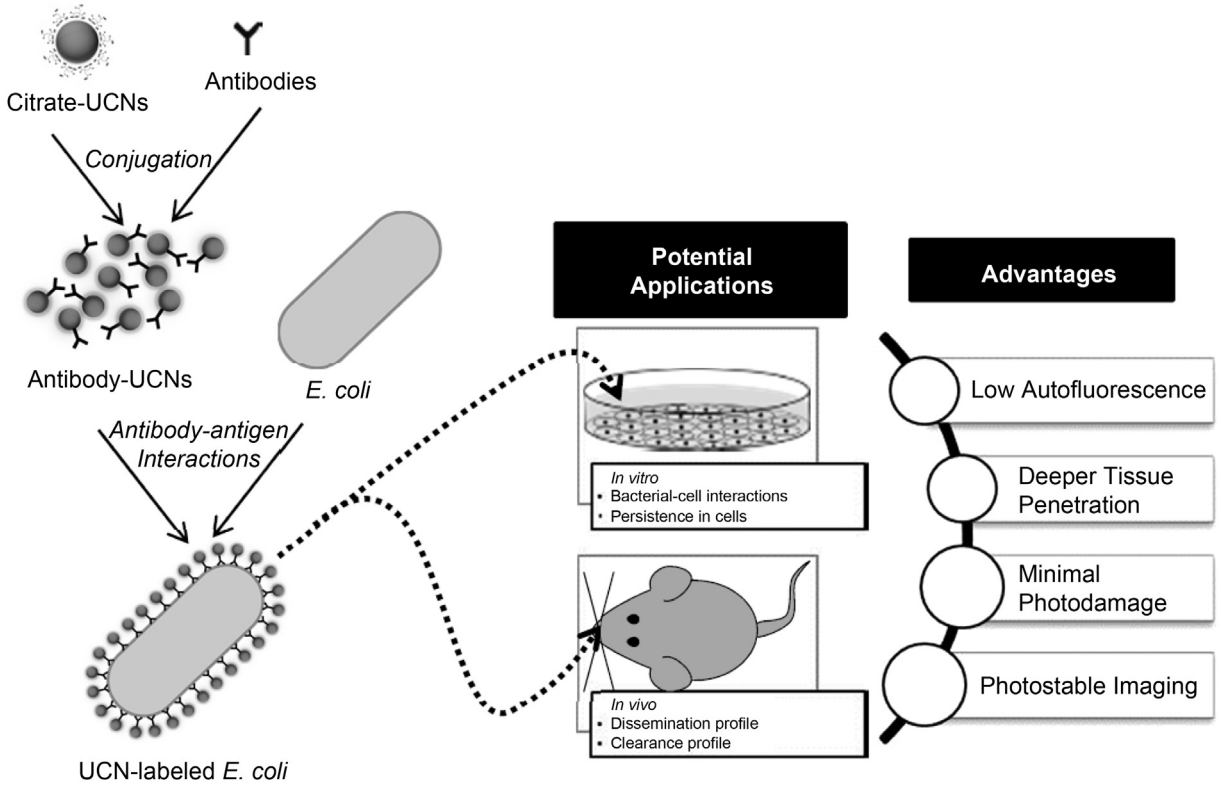

Fig. 1. Schematic diagram illustrating the bacteria labeling strategy to obtain UCN-labeled *E. coli* that can be used for various *in vitro* and *in vivo* studies, while having the advantages of low autofluorescence, deeper penetration, minimal photodamage and photostability.