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Bioengineering novel floating nanoparticles for protein and drug delivery

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Abstract

Gas vesicle nanoparticles (GVNPs) are hollow protein nanoparticles produced by *Halobacterium* sp. NRC-1 which are being engineered for protein delivery. To advance the bioengineering potential of GVNPs, a strain of NRC-1 deleted for the *gvp*C gene (Δ*gvp*C) was constructed and a synthetic gene coding for *Gaussia princeps* luciferase was fused to an abbreviated *gvp*C gene on an expression plasmid. When introduced into theΔ*gvp*C strain, an active GvpC-luciferase fusion protein bound to GVNPs resulted. These results represent both a technical improvement in the GVNP display system and its expansion for the display of active enzymes.

Keywords

Gas vesicle naoparticles (GVNPs); vaccine; luciferase; *Gaussia princeps*; enzyme display

1. Introduction

Gas vesicle nanoparticles (GVNPs) are found in diverse prokaryotic species inhabiting aquatic environments, including both archaeal halophiles and methanogens, and also bacteria. They confer cell buoyancy and function in vertical motility and stratification of photosynthetic cyanobacteria and phototrophic haloarchaea. GVNPs usually have a species-characteristic shape and morphology and range in length from 50 nm to over 1 μm and in width from 30 to 250 nm. Halophilic archaea (haloarchaea) such as *Halobacterium* sp. NRC-1 produce lemon-shaped GVNPs of about 300 nm length and 200 nm width[1].

GVNPs are easily purified from *Halobacterium*sp. NRC-1 byosmotic lysis and centrifugally accelerated flotation [2]. The nanoparticles consist of a thin (20 Å) and extremely stable, lipid-free membrane composed solely of protein bounding a gas-filled space. The membrane is gas-permeable and allows the diffusion of many dissolved gases, which appear to be in

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equilibrium with those in the cytoplasm [3]. GVNP synthesis proceeds by the growth of small bicones to progressively larger lemon-shaped structures. During this process, water is thought to be excluded due to hydrophobicity at the inner surface of the membrane.

Genetic analysis of *Halobacterium* NRC-1 showed the requirement in GVNP formation of a gene cluster, *gvp* M, L, K, J, I, H, G, F, E, D, A, C, N, and O (Fig. 1A), on plasmid pNRC100 [4, 5]. Immunoblotting and biochemical analysis showed that GvpA represented the major protein component, and two similar polypeptides, GvpJ and M, were minor components hypothesized to determine the curvature of the vesicle membrane [6, 7]. GvpC protein was found to be bound to the external surface of GVNPs. Three additional proteins, GvpF, GvpG, and GvpL, were also detected, with GvpF and GvpL containing coiled-coil domains, suggestive of self-association, and the GvpL protein showing laddering on gels [7]. GvpF has recently been localized to the conical tips of GVNPs[8].

*Halobacterium*sp. NRC-1 GvpC protein was targeted for bioengineering of GVNPs, after insertions into *gvp*C resulted in production of fusion proteins and smaller nanoparticles[5, 9]. A motif is repeated 7-8 times in the GvpC protein and suggested a role for multidentate binding to GvpA, and promoting growth and strengthening of nanoparticles [6]. Large insertions were tolerated near the C-terminal end of GvpC and resulted in the display of foreign sequences on the GVNP surface[10]. A series of studies with antigenic proteins from diverse pathogens, including SIV, *Chlamydia*, and *Salmonella*, showed that GvpC-antigen fusion proteins were efficiently displayed to the immune system [5,9-16].

Recently, we have been working to enhance the genetic system for production of GVNPs displaying diverse antigenic and therapeutic proteins (Fig. 1B) [14-16]. In this report, we demonstrate improvement in the GVNP bioengineering system and its utilization for the display of an active enzyme on the surface.

2. Materials and methods

*2.1. Halobacterium*ΔgvpC strain construction

Approximately 500bp regions flanking *gvp*C were PCR amplified using primers with *Hin*dIII sites and cloned into the *Hin*dIII site of pBB400 [17]. The resulting plasmid, pBB400Δ*gvp*C, was used to delete 359/382 codons of the *gvp*C gene, with seven additional codons inserted in the crossover region. The plasmid was used to transform *Halobacterium* sp. NRC-1Δ*ura*3 and pBB400Δ*gvp*C transformants were selected using standard procedures[17,18]. Genomic DNA was extracted and used to screen for knockout candidates by PCR amplification with primers flanking the *gvp*C gene [17, 18].

2.2. Gaussia princeps *Luciferase synthesis and expression in* Halobacterium

In order to express the *Gaussia princeps* luciferase enzyme as a fusion-protein with the C3-portion of GvpC in *Halobacterium* sp. NRC-1 Δ*ura*3Δ*gvpC* strain, we designed a codon-optimized gene (Sequence ID:gb|AAG54095.1) using an in-house Visual Basic script. The script employs a codon usage table for predicted genes in the fully sequenced *Halobacterium* sp. NRC-1 genome to replace rare codons with highly used codons [19]. The 185 amino acid-long synthetic gene was generated commercially, flanked by *Afe*I cloning sites (Life

Technologies, Carlsbad, CA), and cloned into the pDRK-C3 overexpression vector at an *Afe*I site downstream from the *gvp*A promoter, followed by sequencing to verify the correct sequence [14]. The resulting plasmid, pDRK-C3-*luc*, encoded a His-tagged fusion protein, arranged as His_6-GvpC3-Luc, and was transformed into the *Halobacterium* sp. NRC-1 Δ*ura*3Δ*gvp*C strain, for enzyme display on the exterior of GVNPs. Expression of the correct protein in the resulting strain, *Halobacterium* sp. NRC-1 Δ*ura*3Δ*gvp*C (pDRK-C3-*luc*), was confirmed by Western blotting using an anti-His-tag antibody (Cell Signalling Technology, Danvers, MA) and an alkaline phosphatase-conjugated goat antirabbit secondary antibody (Sigma-Aldrich, St. Louis, MO).

2.3. GVNP preparation

The *Halobacterium* sp. NRC-1 Δ*ura*3Δ*gvp*C (pDRK-C3-*luc*)Luc-GVNPs were produced and purified by overnight floatation of the GVNPs using the centrifugally accelerated procedure previously reported [14].

2.4. Luciferase assays

Purified gas vesicle nanoparticles were assayed for *Gaussia princeps* luciferase activity using the Glow Assay system per manufacturer's instructions (Thermo Scientific). Assays were conducted in 96-well microtiter plates using a SpectraMax M5 luminometer (Molecular Devices, Sunnyvale, CA). Induction was calculated in relative light units (RLU) of the treated sample/average relative light units of the untreated samples.

3. Results

3.1. Δgvp*C-strain construction*

The genetic system currently in use for bioengineering of GVNPs has been technically challenging due to the large size and complexity of the *gvp* gene cluster used to complement the *Halobacterium* host strain. In order to facilitate the genetic system for bioengineering GVNPs, we constructed a new *Halobacterium* sp. NRC-1 derived host (*Halobacterium* sp. NRC-1Δ*ura*3Δ*gvp*C)deleted only for the *gvp*C gene within the *gvp* gene cluster (Fig. 1A). The *gvp*C deletion constructed was verified by PCR amplification of the deleted region (data not shown). This strain can serve as the complementation host for a smaller expression plasmid containing a portion of the *gvp*C gene, for expression and display of foreign proteins on the GVNPs.

3.2. gvp*C3 expression plasmid*

For complementing the *Halobacterium* sp. NRC-1Δ*ura*3Δ*gvp*C strain, we constructed a derivative of the previously described pDRK expression plasmid containing a fragment of the *gvp*C gene (Fig. 1B) [20]. This pDRK-C3 plasmid contains a sequence encoding the C3 fragment of GvpC (280 out of 382 amino acids) downstream of the *gvp*A promoter and His_6 coding region. Plasmid pDRK has been shown to direct the high level expression of other proteins in previous work [20, 21]. The pDRK-C3 plasmid construct contains a mevinolin-resistance gene for selection in *Halobacterium*, an ampicillin-resistance gene for selection in *E. coli*, and a unique *Bam*HI restriction site for construction of fusion genes.

3.3. Expression of GvpC-Luciferase

A synthetic luciferase reporter gene from *Gaussia princeps* was inserted at the 3' end of the *gvp*C gene fragment in the pDRK-C3 plasmid, to construct pDRK-C3-*luc*. pDRK-C3-*luc* was transformed into *Halobacterium* sp. NRC-1Δ*ura*3Δ*gvp*C to construct the expression strain *Halobacterium* sp. NRC-1Δ*ura*3Δ*gvp*C (pDRK-C3-*luc*) (Fig. 1B).

The *Halobacterium* sp. NRC-1Δ*ura*3Δ*gvp*C (pDRK-C3-*luc*) expression strain produced an enzymatically active GvpC-luciferase fusion protein bound to GVNPs. This was shown by purification of the GVNPs via centrifugally accelerated flotation overnight and using the luciferase glow assay [14] (Fig. 2). The luciferase activity was stable to freezing and thawing (Fig. 2) as well as heating (data not shown). These findings showed that the improved genetic system could be used for displaying an enzyme on *Halobacterium* GVNPs and that the displayed enzyme was stable to low and high temperatures.

4. Discussion

In this report, we have documented an improved genetic system for displaying foreign proteins on GVNPs. The display system streamlines the expression plasmid from a large (~20 kb) one containing the entire *gvp* gene cluster to a much smaller plasmid, pDRK-C3 (9 kb)limited to the *gvp*C3 fragment, and a host strain, *Halobacterium* sp. NRC-1Δ*ura*3Δ*gvp*C, deleted solely for the *gvp*C gene. This genetic system provides a technically simpler system for introduction of foreign sequences and production of GVNPs displaying GvpC-fusion proteins.

We have tested the newly developed genetic system using the luciferase reporter from the marine copepod, *Gaussia princeps*. A synthetic codon-optimized luciferase gene was inserted into the pDRK-C3 expression plasmid in order to construct pDRK-C3-*luc*, which when transformed into the *Halobacterium*Δ*gvp*C strain, resulted in the production of the active enzyme bound to GVNPs. This system could also be shown to produce GVNPs with both wild-type GvpC protein and GvpC-Luc fusion protein by transforming pDRK-C3-*luc* into *Halobacterium* sp. NRC-1 (data not shown). This additional finding demonstrated that multiple GvpC proteins may be displayed on the nanoparticles.

GVNPs provide a remarkably stable and buoyant nanoparticle platform for bioengineering. The GvpC fusion system represents a novel method for displaying proteins as a two-dimensional array on the external surface of the nanoparticles in a form which allows display and delivery to the mammalian immune and vascular systems[1, 5, 9-13, 15, 16]. As a result of the biocompatibility and the extreme stability of the nanoparticles, the potential of GVNPs is likely to be very significant. The ability of GVNPs to deliver multiple antigens and enzymes, and their scalability and safety, extend the potential utility of these novel nanoparticles.

5. Conclusion

GVNPs are remarkably stable and buoyant nanoparticles. We have improved the genetic system for their bioengineering and demonstrated their value as a method for displaying a

new protein, the easily assayed enzyme, luciferase. As a result of the biocompatibility, scalability, safety, and bioengineerability, GVNPs represent a significant resource for biotechnology.

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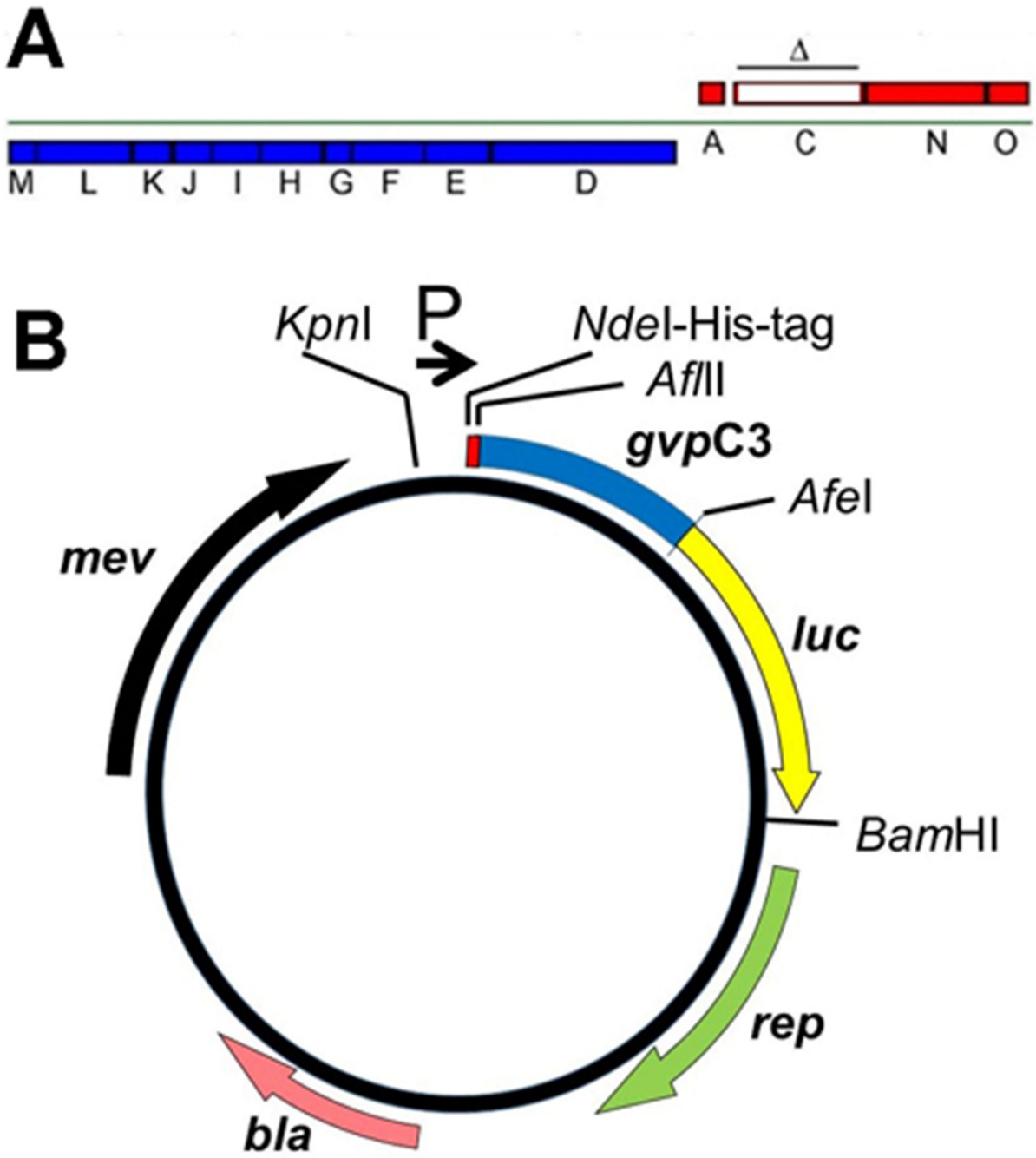

Figure 1.

Construction of an improved genetic system for expression of foreign proteins fused to GvpC and display on GVNPs. A. *Halobacterium* sp. NRC-1 gene cluster for synthesis of GVNPs, showing leftward transcribed genes in blue and rightward transcribed genes in red and white. The *gvp*C gene was deleted in *Halobacterium* sp. NRC-1 Δ*ura*3Δ*gvp*C, with the deleted region labelled Δin white. B. Plasmid pDRK-C3-*luc* with locations of genes marked by circular arrows (*bla*, β-lactamase; *mev*, mevinolin-resistance; *gvp*C3, coding for C3

fragment of GvpC, *luc*, codon-optimized luciferase gene, *rep*, replicase), promoter marked P, and location of restriction sites indicated.

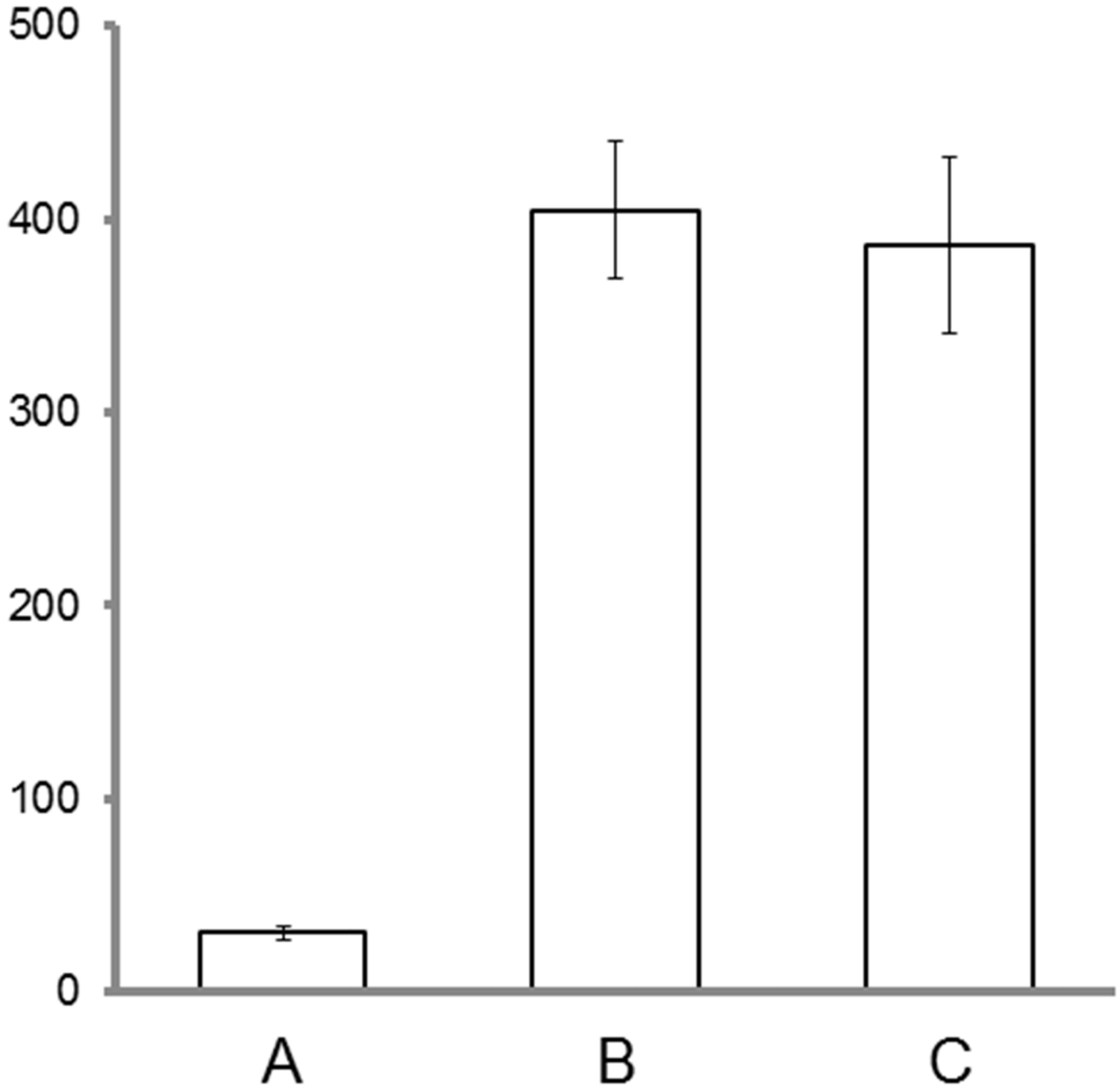

Figure 2.

Luciferase activity in strain *Halobacterium* sp. NRC-1Δ*ura*3Δ*gvp*C (pDRK-C3-*luc*). A. Background signal. B. Signal in GVNPs purified by flotation. C. Signal after freezing and thawing. Standard deviations of 4 assays are shown with error bars indicated. Y Axis: 10^3 RLU.