

## Nanoparticle Hydrophobicity Dictates Immune Response

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### Supporting Information

**ABSTRACT:** Understanding the interactions of nanomaterials with the immune system is essential for the engineering of new macromolecular systems for in vivo applications. Systematic study of immune activation is challenging due to the complex structure of most macromolecular probes. We present here the use of engineered gold nanoparticles to determine the sole effect of hydrophobicity on the immune response of splenocytes. The gene expression profile of a range of cytokines (immunological reporters) was analyzed against the calculated log *P* of the nanoparticle headgroups, with an essentially linear increase in immune activity with the increase in hydrophobicity observed in vitro. Consistent behavior was observed with in vivo mouse models, demonstrating the importance of hydrophobicity in immune system activation.

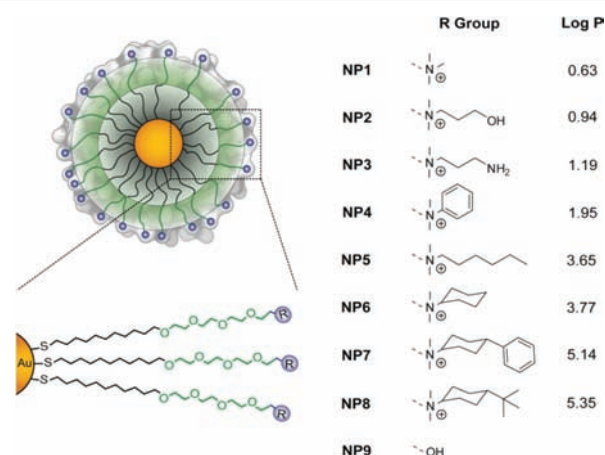
Navigating the response of the immune system is a major issue in the design of nanomaterials for in vivo applications. For example, avoiding immune system detection is an important consideration in gene and drug delivery,<sup>1</sup> whereas in the case of adjuvants for vaccine therapies, immune activation is desired.<sup>2</sup> Therefore, a deeper understanding of how nanomaterials elicit immune responses is essential for the optimization of these systems for biomedical applications.<sup>3</sup>

A key issue in understanding immune system activation by macromolecular probes is determining interactions of these materials with the *innate* immune system, the first line of defense of the body and the gatekeeper to full immunoresponse.<sup>4</sup> Innate immune activation is associated with the recognition of conserved molecular motifs related with pathogens (pathogen-associated molecular patterns, PAMPs)<sup>5</sup> as well as nonspecific danger-associated molecular patterns (DAMPs).<sup>6</sup> Hydrophobicity per se is considered to be a DAMP.<sup>7</sup> Under healthy conditions, hydrophobic cellular materials (“hypos”) are hidden from the external environment. During necrotic cell disruption or protein denaturation, however, these hypos become exposed, and by interaction with membranes and specific surface receptors, an innate immune response is generated. This response has been hypothesized to be the origin of the need for oil-based adjuvants in vaccine treatments.<sup>8</sup>

Quantifying the relationship between hydrophobicity and immune response is experimentally challenging. In aqueous

environments, structural changes and aggregation accompany variations in the hydrophobic content of synthetic<sup>9</sup> and biomolecular agents (e.g., proteins and lipids).<sup>10</sup> As a result, immune response to the hydrophobicity of these materials is also influenced by structural differences in the probe, complicating the structure–activity correlation of these systems.<sup>11</sup>

In recent studies, nanoparticles with well-defined surfaces have been used to probe the interactions of nanomaterials with biological systems.<sup>12</sup> We have developed a family of gold nanoparticles (AuNPs, Figure 1) designed to explore



**Figure 1.** Chemical structure of the monolayer-protected 2-nm core diameter gold nanoparticles. The passivating tetra(ethylene glycol) spacer (green area) removes possible background effects from the nanoparticle hydrophobic interior (gray zone). To generate the profiles for the SAR studies, functionalities (blue) are tuned at the ligand termini to control the surface hydrophobicity. Log *P* represents the calculated hydrophobic values of the headgroups.

structure–activity relationships (SAR) at biological interfaces.<sup>13</sup> A key point in the design of these AuNPs is the use of a passivating noninteracting tetra(ethylene glycol) spacer to remove background effects arising from the core and hydrophobic ligand interior, while also preventing aggregation even in complex biofluids such as serum.<sup>14</sup> Using these particles, different functional groups can then be displayed at the AuNP surface and their effects isolated using this inherently

Received: November 19, 2011

Published: February 17, 2012

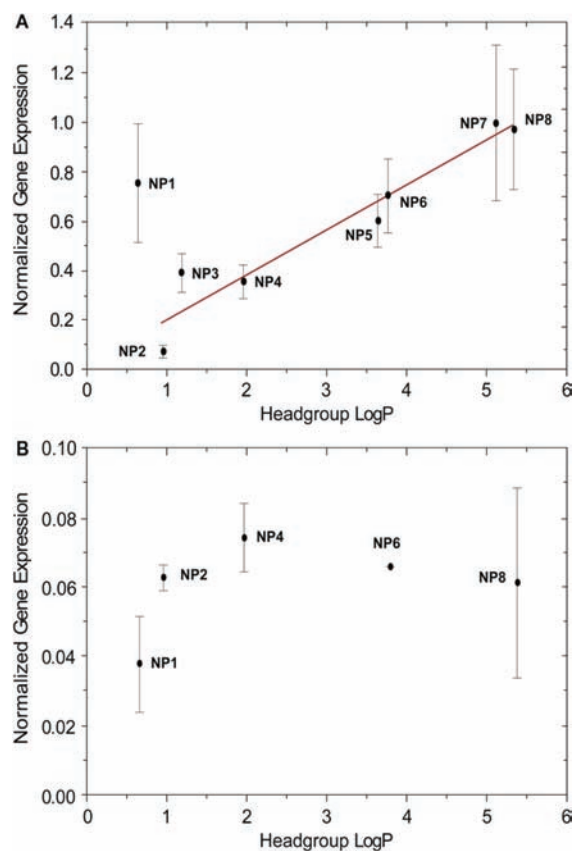
multivalent platform. This design hence provides structural uniformity, thus offering means to utilize specific surface attributes for SAR purposes.<sup>15</sup> We report here the use of this AuNP model to quantify the interplay between hydrophobicity and immune activation of splenocytes.

SAR studies at the nanomaterial level provide an efficient tool in the analysis of nanoparticles properties.<sup>16</sup> When other structural parameters are controlled, nanoparticle properties can be described and established on the basis of descriptors of their surfaces.<sup>17</sup> Given that NP1–8 differ only in their surface functionality (Figure 1) and their physicochemical properties are similar (both at room temperature and at 37 °C, Figures S3, S4, S5, and S6 in Supporting Information [SI]), we used the computationally predicted *n*-octanol/water partition coefficient<sup>18</sup> of the ligand headgroup (R groups, Figure 1) as the quantitative descriptor of relative nanoparticle surface hydrophobicity. Log *P* values were estimated using MacroModel (Maestro 8.0).<sup>19</sup> The calculations were performed at 298 K using the Merck Molecular Force Field (MMFF94).

In our initial studies we explored the immune response of NP1–8 with splenocytes, profiling cytokine mRNA levels to provide a direct assessment of immune response.<sup>20</sup> This measurement can be done since protein expression follows gene expression for the cytokines under study.<sup>21</sup> Splenocytes were selected as the experimental model of study as they are the reservoir of immune cells packed in the largest lymphoid organ in the body. These cells are comprised mostly of B-lymphocytes, but also include T cells and monocytes.<sup>22</sup> Taken together, these jointly represent both the innate and the adaptive arms of the immune system.<sup>23</sup> Splenocytes harvested from mice were exposed to each nanoparticle (10 μM) under in vitro conditions. After 2 h, the cells were washed and lysed. Quantitative RT-PCR was employed to quantify the mRNA expression level associated with each one of the cytokines; primers for IL-2, IL-6, IL-10, TNFα, IFNγ, and the interferon responsive genes OAS1, STAT1, and IFNβ were used to preferably amplify them from the cDNA library and normalized against housekeeping genes HPRT1 and GAPDH.<sup>20</sup>

As shown in Figure 2A, the plot of cytokine expression against log *P* reveals an essentially linear correlation between hydrophobicity and immune response, with the exception of NP1. This trend was observed for each of the cytokines under study, with variations only observed in the relative level of expression (Figure S1), indicating a selective type of immune response.<sup>24</sup> The distinct behavior of NP1 can be explained by its highly exposed charge, capable of inducing alternate responses through electrostatic interactions<sup>15</sup> or by contact with specific amino acids.<sup>25</sup>

In vivo response to nanomaterials is much more complex than in vitro systems.<sup>26</sup> We probed immune response to NP1–8 using mouse models. For that purpose, mice (12 weeks old) were injected intravenously (100 μL) via the tail vein. Each group of mice (*n* = 6 mice per group) received a single dose of a specific nanoparticle at 5 mg/kg. At 1.5 and 6 h post-IV administration, the mice were sacrificed and splenocytes harvested and treated as before to assess cytokine mRNA expression levels. Figure 2B presents the tendency of cytokine expression against log *P* in vivo. At lower log *P* values, increasing hydrophobicity elicits increased immune response. However, with high degrees of hydrophobicity the dependence is less evident, and a maximum in immune response is observed. This leveling off can be explained by the expected changes in biodistribution for hydrophobic nanoparticles, in



**Figure 2.** Cytokine gene expression as a function of nanoparticle headgroup log *P*. (A) TNFα (a representative pro-inflammatory cytokine) in vitro gene expression and (B) IL-10 (a representative anti-inflammatory cytokine) in vivo gene expression as a function of the calculated AuNP headgroup log *P*. The gene expression values are normalized by dividing the observed response against the values of a positive control (LPS) under the same experimental set. A minimally interacting neutral particle (NP9) was used as a negative control. NPs were used at a concentration of 10 μM for in vitro and 5 mg/kg for in vivo studies. Data were taken 2 h (in vitro) and 1.5 h (in vivo) after exposure to AuNPs. In vivo correlation is lost at 6 h (Figure S2).

particular the poor biodistribution expected for highly hydrophobic particles.<sup>27</sup> Nonetheless, it is clear that, upon availability of hydrophobic portions in the system, immune response is generated (Figure S2, correlation is lost at 6 h time).<sup>24</sup>

In summary, we have demonstrated a direct, quantitative correlation between hydrophobicity and immune system activation, an important determinant for nanomedical and nanoimmunological applications. This correlation provides a promising starting point for determining the specific molecular mechanisms of immune cell activation,<sup>28</sup> an issue of importance for understanding the evolution of the innate immune system.<sup>29</sup> Moreover, these probes present both a tool for harnessing the immune system and a probe for quantifying the role of hydrophobicity in immune response.<sup>30</sup>

## ■ ASSOCIATED CONTENT

### § Supporting Information

In vitro and in vivo cytokines expression plots, and nanoparticles characterization data (DLS, TEM, ZP). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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## Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

This work was supported by grants from the ISF (#181/10), the Lewis foundation for blood cancer, and the Kenneth Rainin Foundation to D.P. V.M.R. acknowledges support from the NIH (GM077173) and the Center for Hierarchical Manufacturing (CMMI-1025020).

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