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A Parallel Approach to the Discovery of Carrier Delivery Vehicles To Enhance Antigen Immunogenicity

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A consistent obstacle to the therapeutic exploitation of carbohydrate-based tumor-associated antigens is their poor immunogenicity in vivo. Thus, carbohydrate-derived vaccines are often ineffective at triggering malignant cell destruction. Consequently, new strategies are being sought to improve the mammalian immune system's ability to recognize and target these key cancer cell-surface motifs.[1]

The concept of using polymeric controlled-release technology to improve vaccine efficacy was first demonstrated by Langer in 1979.[2] Since that time numerous reports have demonstrated the enhanced immunogenicity of encapsulated versus nonencapsulated antigens.[3] However, the general utility of polymeric materials has been limited in part because of the link between the structure of the encapsulating polymer, and improvement of antigen immunogenicity is difficult to predict.

The ganglioside GM_3 **1** is expressed in abnormally high levels in tumor cells[4] and its poor immunogenicity is well documented (Figure 1a).[5] As part of an ongoing effort to generate human[6] and murine monoclonal antibodies against such tumor-associated antigens we have merged the rapidly expanding disciplines of parallel polymer synthesis[7] and controlled-release technology with immunology to produce a rapid and generic approach to improve the immunogenicity of carrier-bound antigens.

The process involves three stages: first, an array of cross-linked hydrogel materials containing a carrier protein (at various concentrations) is prepared in parallel in one step; second, the array is screened in mice to determine the most effective hydrogel at enhancing the immunogenicity of the encapsulated versus nonencapsulated carrier; and finally, the most efficient hydrogel is prepared containing the critical carrier−antigen conjugate and is used for immunization protocols. The rational for encapsulation of the carrier (in stage 1) rather than the antigen−carrier conjugate is driven by the current and future needs to conserve limited amounts of valuable and often costly immunogens.

The strategy was tested by preparing poly(acrylamide) hydrogel arrays derived from monomer **2** and cross-linkers **3a**,**b** (at various mole percent), in which bovine serum albumin (BSA) is encapsulated (Figure 1b). The choice of BSA was guided by its ubiquitous role as a carrier during immunization protocols. Screening the array in vivo highlighted the most capable hydrogel at elevating the serum titers of anti-BSA immunoglobulin G (IgG). The synthetic glycoconjugate BSA-**4** was then encapsulated into this key hydrogel and the power of this approach was reflected in the significantly elevated serum anti-**4** IgG antibody titers produced following immunization with the encapsulated BSA-**4**.

Poly(acrylamide)s were considered plausible candidates for array synthesis because they are easily prepared in one step via radical polymerization of an acrylamide and cross-linker. The monomers are readily available and multi-functional cross-linkers can be easily synthesized. In addition, poly(acrylamide)s are relatively nontoxic and as such their role as antigen delivery vehicles should not be limited by acute toxicity.[8]

Polymerization reactions were conducted in parallel in individual glass vials. The reactions involved a bulk polymerization process employing monomer **2** and varying concentrations of cross-linkers **3a**,**b** (1, 1.6, 2.0, 3.8, 5.7, and 7.4 mol %) and two concentrations of the carrier protein BSA (0.9 and 0.4 mg/mL).[9] A priori, cross-linking agents investigated for poly(acrylamide) synthesis have contained two functional groups (reactive olefins) with only few examples of more than two such functional groups. Therefore, cross-linker **3b** was included in the array to probe the effects of both di- and trifunctional cross-links in the poly(acrylamide) network.[10] A mild redox polymerization system was used to minimize protein modification.[11] Under these conditions, polymerizations were typically complete within 1 h and the integrity of the encapsulated BSA was not compromised (as confirmed by SDS-gel electrophoresis, data not shown). The final array was comprised of 24 members ($1 \times 2 \times 6 \times 2$) and yielded between 200 and 400 mg of each material.[12]

The antigen delivery property of the hydrogel array was screened in parallel in a mouse model. The hydrogels were administered as a suspension, via intraperitoneal (*i.p.*) injection with an adjuvant (RIBI), in PBS. A booster injection, identical in constitution to the first injection, was administered on day 14 and serum samples were tested for IgG antibody titers to BSA on day 17 (by ELISA, Figure 2).

The data reveal a significant improvement in the immunogenicity of BSA encapsulated in hydrogels, both at high and low carrier concentrations. Three members of the array caused a significant elevation in the immunogenicity of BSA at the 90 μg administration dose above that of nonencapsulated BSA (titer $= 3200 \pm 400$) (Figure 2a). All the effective hydrogels contain the **3a** cross-link [either at 3.8 (titer $= 7800 \pm 1800$), 1.6 (titer $= 7500 \pm 2000$), or 1.0 (titer 7200 ± 2000) mol %]. The most profound improvement is observed when BSA administration occurs at the lower concentration (40 μg) (Figure 2b). All the hydrogels tested produce an anti-BSA antibody titer significantly greater than that for the nonencapsulated BSA (titer $= 200 \pm 100$, $n = 8$).[13] However, the most efficient hydrogels are derived from the trifunctional cross-linker **3b**, with antibody titers exhibiting a good correlation with reciprocal cross-link percent across the array. The observations that hydrogels cross-linked with **3b** rather than **3a** would be more effective as controlled-release vehicles (at low BSA concentration) and that the inverse relationship between cross-link percent and serum titer is not readily predictable, based on the cross-linkers'

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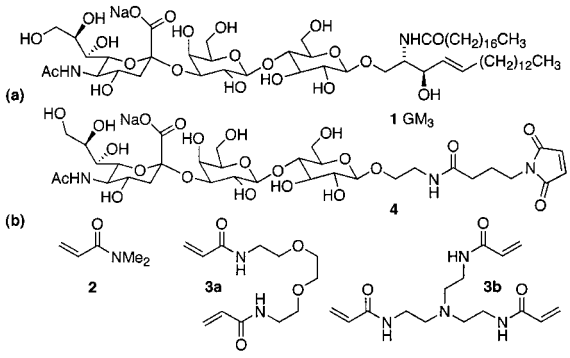

Figure 1. (a) To enhance the immunogenicity of GM_3 **1** a BSA glycoconjugate of analogue **4** was encapsulated within the most efficient hydrogel in (b). (b) Poly(acrylamide) hydrogel arrays were prepared from monomer **2** and cross-linkers **3a**,**b**.

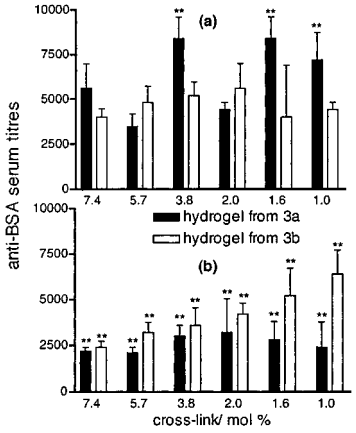

Figure 2. Anti-BSA IgG serum titers, determined by ELISA, following parallel immunization with hydrogel vehicles. (a) Immunization with encapsulated BSA (90 μg). (b) Immunization with encapsulated BSA (40 μg). Each point is reported as a mean value ($n = 4$) $\pm$ SEM and significance is relative to serum titers of nonencapsulated BSA at the same dose (the double asterisk (**) indicates $P < 0.05$).

structure alone, thus illustrates the utility of this parallel approach for nonintuitive lead identification.

Based on these results the hydrogel comprised of 1.0 mol % **3b** was selected to be the delivery vehicle for the BSA-**4** glycoconjugate. The attenuated GM_3-derivative **4** was synthesized by taking advantage of literature procedures.[14] BSA and ovalbumin (OVA) glycoconjugates of **4** were prepared and MALDI-TOF mass-spectrometry was used to confirm the successful conjugation. The BSA-**4** conjugate was then encapsulated within the **3b** (1.0 mol %) hydrogel as described, vide supra. Mice (129 GiX^+, 4-per group) were immunized (on day 1 and day 14) with either the nonencapsulated BSA-**4** conjugate or the encapsulated BSA-**4** (equivalent to 40 μg of carrier). Mean serum anti-OVA-**4** IgG antibody titers (day 21) confirmed the success of the encapsulation strategy (encapsulated BSA-**4** 3200 $\pm$ 200; nonencapsulated BSA-**4** no detectable titer).

Parallel synthesis and in vivo screening of poly(acrylamide) hydrogels is a rapid and efficient method for highlighting optimal delivery vehicles for carrier-supported antigens. We have applied our strategy to generate a hydrogel material that significantly elevates the immunogenicity of a BSA-glycoconjugate of the truncated tumor-associated antigen GM_3 analogue **4**. This method should have broad applicability to immunization strategies where the antigen is a weak immunogen or available only in limited amounts and should be suitable for all carrier proteins.

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Supporting Information Available: Experimental details of **3b** and hydrogel array synthesis, immunization protocols, serum titer measurement by ELISA, and glycoconjugate BSA-**4** preparation (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) For a review discussing the low toxicity of poly(acrylamide)s see: King, D. J.; Noss, R. R. *Rev. Environ. Health* **1989**, *80*, 3−16. We have also observed that a poly(acrylamide) hydrogel, based on monomer **2** and cross-linker **3a**, has no observable acute toxicity against a human dermal fibroblast cell line (data not shown).
(9) Different protein concentrations were investigated because modulation of internal osmotic pressure of the protein within the hydrogel is an additional variable in controlling carrier release.
(10) Cross-linker **3a** was prepared as described previously (see: Nagahori, N.; Nishimura, S. I. *Biomacromolecules* **2001**, *2*, 22−24). Cross-linker **3b** was synthesized (in 72% yield) by treatment of tris(2-aminoethyl)amine with acryloyl chloride and was found to be water soluble.
(11) Ammonium persulfate/*N*,*N*,*N*′,*N*′-tetramethylenediamine neutralized to pH 7.0 in water at 0 °C.
(12) The hydrogels were opaque solids and were easily ground into a fine powder for immunization. Unreacted monomer **2** and cross-linker **3a**,**b** were removed from the hydrogel particles by rinsing with phosphate buffered saline [PBS (100 mM sodium phosphate, 50 mM NaCl, pH 7.4)]. Analysis of the PBS washings revealed that all monomer **2** and **3a**,**b** were removed prior to immunization. Release of BSA from the hydrogels exhibits a biphasic profile (please refer to the Supporting Information for full details), with a rapid initial release phase (up to ca. 24 h for cross-linker **3a** and 15 h for **3b**) followed by a slower second phase. The extent and hence amount of BSA released in the initial phase is inversely related to the amount of **3a**,**b** present in the network.
(13) The source of the hydrogels' ability to enhance the carrier's immunogenicity is not linked to possession of intrinsic adjuvant activity. Additional immunization studies were undertaken in parallel (in the absence of RIBI adjuvant) with the hydrogel array and no serum anti-BSA antibody titers were detected.
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