Dendrigraft Poly-L-lysine: A Non-Immunogenic Synthetic Carrier for Antibody Production

Bernard Romestand,[†] Jean-Luc Rolland,[†] Auguste Commeyras,[‡] Gaëlle Coussot,[§] Isabelle Desvignes,[§] Robert Pascal,[§] and Odile Vandenabeele-Trambouze*[,§]

IFREMER, Université Montpellier 2, Centre National de la Recherche Scientifique, Unité Mixte de Recherche 5119, Ecosystèmes Lagunaires, place E. Bataillon, CC80, 34095 Montpellier cedex 5, France, COLCOM, Cap-Alpha, Av. de l'Europe, Clapiers, 34940 Montpellier Cedex 9, and Institut des Biomolécules Max Mousseron, Centre National de la Recherche Scientifique, Université de Montpellier 1, Université de Montpellier 2, Unité Mixte de Recherche 5247, place E. Bataillon, CC17006, 34095 Montpellier cedex 5, France

Received October 23, 2009; Revised Manuscript Received April 7, 2010

An easily synthesized DendriGraft poly-lysine DGL-G3 (third generation) was shown to act as an efficient carrier for raising antibodies directed against small molecules. The immunological properties of three different forms of DGL-G3 were investigated: the native form (molecular weight 22 kDa bearing a mean number of 123 surface amino groups as TFA salts), a form modified at the C-terminus by fluorescein (fluorescein-DGL-G3), and last a surface-modified form bearing histamine (DGL-G3-Histamine). Our studies demonstrate the native DGL-G3 to be inefficient in eliciting antibody production in rabbits. Immunizations of rabbits using the core-modified fluorescein-DGL-G3 or the surface-modified DGL-G3-histamine conjugate failed in eliciting antibody production. Conversely, following a primary immunization using a BSA-histamine conjugate, a second immunization with DGL-G3-histamine conjugate improved the production of specific hapten-directed antibodies, which demonstrates the utility of DGL-G3 as a carrier for the production of highly specific antibody against haptens.

Introduction

The use of synthetic peptide antigens for the production of vaccines[1,2] as well as the use of haptens for the production of antibodies for diagnostic immunoassays[3−14] suffers the problem of finding carriers devoid of side effects.[14] An advantageous carrier should be poorly (or preferentially non) immunogenic, allow or enhance the immune response specifically toward peptides or haptens, and also bear multiple immunogenic targets. Consequently, the carrier should present many functional groups (e.g., amines, carboxylic acids) easy to derivatize and resist to the denaturation by the organic solvents potentially needed during the immunoconjugate synthesis process. Moreover, it must be nontoxic, inexpensive, and the final conjugate must be water-soluble.

All known natural carriers (e.g., BSA, KLH, HSA) are strongly immunogenic.[15] Synthetic carriers like poly-[Lys-(D,L-Ala)^3] or poly tuftsins are less immunogenic but produce a weak immune response compared with KLH or BSA.[16−19] Linear poly-L-lysines (PLL)[20] and polyaminoamide dendrimers (PAMAM)[21] show a weak (but non-null) intrinsic immunogenicity. So far, the dendrons of L-lysine are synthetic nonimmunogenic carriers, a property that prompted their utilization in the synthesis of multiple antigen peptides (MAPs) (Scheme 1A) designed to be used for the production of synthetic vaccines.[1,2,23−26] Following this aim, nonimmunogenic linkers should preferably be selected to anchor the antigen to the carrier. For vaccine production, the most direct strategy to obtain conjugates is to build a MAP carrier−peptide construct in the synthesis stage by means of the solid-phase method.[22,27] Anchorage is then simply ensured by a peptide bond. This technique of synthesis is limited to epitope-like peptides with molecular weights (MWs) lower than 4 kDa. This limitation is the result of the accumulation of deletions, truncations, and side-reactions along the numerous steps of synthesis.[28,29]

An alternative strategy to prepare MAPs is to use homogeneous phase methodology. In this case, MAPs are finally obtained by ligation of pure peptide epitopes (independently synthesized, purified, and analytically controlled) to a nonimmunogenic carrier with a targeted MW around 20 kDa. In this case, the challenge is to connect the epitope to the synthetic carrier by a nonimmunogenic linker. This strategy has been developed by several groups.[30−37]

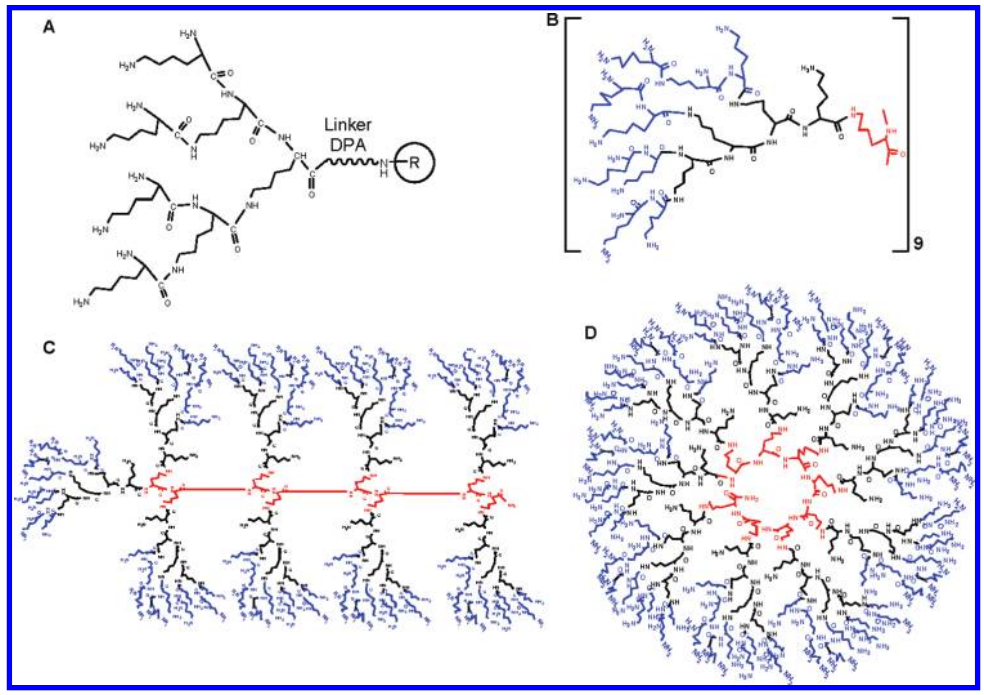
Similar strategies can be used in the field of antibody production for diagnostic immunoassays. The difficulty lies in the linker choice used to attach the hapten to the carrier. Its intrinsic immunogenicity must be balanced. In a previous work, we developed a successful strategy using two different types of carriers;[3−5] the two primary immunizations involved a highly immunogenic natural bovine or human serum albumin and the third one involved a linear poly-L-lysine, which is less immunogenic but not completely furtive. A reduced glutaraldehyde was chosen as a linker leading to highly specific and affine antibodies directed against the hapten.[3−5]

In this present work, our objective was to find a truly nonimmunogenic carrier with a minimum MW around 20 kDa and a large number of peripheral reactive groups for hapten conjugation.[2,14,15] We chose to study the third generation of dendrigraft poly-L-lysine (DGL-G3), a synthetic polymer with an idealized structure constituted by nine equivalent dendrons (Scheme 1B−D) linked to the core. The core is a linear poly-L-lysine with an average of eight monomers. Each dendron looks like the traditional Tam-type (MAP) dendron synthesized from

* To whom correspondence should be addressed. Tel: +33 (0)467143920. Fax: +33 (0) 467661043. +86-21-34205699. E-mail: o.trambouze@univ-montp2.fr.
[†] IFREMER.
[‡] COLCOM.
[§] Institut des Biomolécules Max Mousseron.

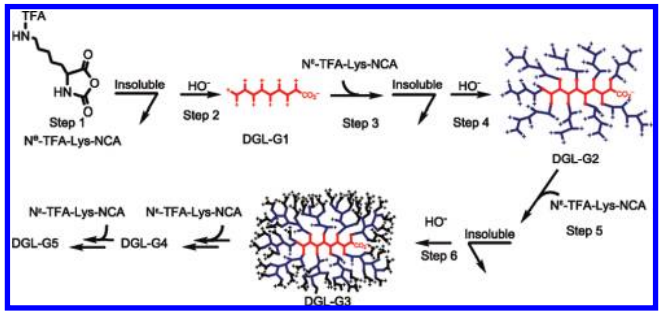
10.1021/bm9012056
Published on Web 04/22/2010

Scheme 1[a]



[a] (A) Traditional Tam-type dendron structure (MAP) of L-lysine synthesized from Merrifield-resins with DPA (2,4-(dimethoxylbenzyl)-phenoxyacetic acid) as a linker. Idealized structure of the same DGL-G3 are displayed. (B) One of the nine idealized dendrons of the overall structure. (C) The full DGL split along the core and (D) the native bulky form considered as globular.[43] Red, blue, and black colors represent the three steps of the DGL-G3 synthesis as described in Scheme 2.

Scheme 2. DGL-G3 Synthesis from N$^{\varepsilon}$-TFA-Lys-NCA Polymerization (Steps 1, 3, and 5)[a]



[a] TFA protecting groups are removed under alkaline conditions (steps 2, 4, and 6). Color code: DGL-G1 (red) and additional lysine molecules grafted on DGL-G1 (blue) and on DGL-G2 (black).

Merrifield resins (Scheme 1 A). DGL-G3 is readily available by synthesis with an MW of 22 kDa.[38,39] Its empirical formula is $K_8K_{115}{}^3 = 123$ with K = lysine, where K_8 is the number of lysines in the core of the dendrigraft; K_{115} is the number of lysines at the interior; 3 is the generation; and 123 is the number of terminal amino groups. DGL-G3 has the structure of a dendrigraft polymer. Such dendrigraft structures were first described by Tomalia[40] and Gauthier[41,42] with monomers different from amino acids.

Here we report on the DGL-G3 immunogenic properties for three different forms (the native form and two modified forms), establishing the utility of DGLs as carriers in raising antibodies against hapten for diagnostic immunoassays.

Experimental Section

Chemicals. The third generation of native DGLs TFA salts (Scheme 1) and core-labeled with fluorescein (fluorescein-DGL-G3 or fluorescein-$K_8K_{115}{}^3$=123) were supplied by COLCOM (Clapiers, France) as a gift and used without further purification. New Zealand female rabbits were purchased from Charles River (Saint-Aubin-lès-Elboeuf, France) and histamine dihydrochloride was purchased from Acros Organic (Noisy-le-Grand, France). The 25% aqueous solution of glutaraldehyde was acquired from JT Baker (Paris, France). Bovine serum albumin fraction VI (BSA) was bought from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Sheep antirabbit immunoglobulins G (IgG) labeled with horseradish peroxidase and *ortho*-phenylene diamine (OPD) were purchased from Bio-Rad (Marnes-la-Coquette, France).

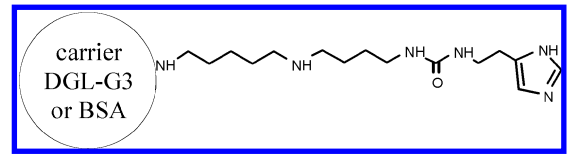


Figure 1. Schematic representation of DGL-G3-histamine or BSA-histamine structures.

Immunoconjugates Synthesis. BSA-histamine and DGL-G3-histamine (Figure 1) were synthesized according to a previously described method.[3−5] In brief, derivatized histamine was coupled to BSA or DGL-G3-TFA salt by glutaraldehyde. The resulting imine intermediates were reduced to stable covalent bonds using borohydride according to a previously described method.[44−46]

Immunization Protocol. Eight New Zealand female rabbits were prebled at day 0 preceding immunization. Then, rabbits were subcutaneously inoculated with 1 mL of a solution containing 150 μg of immunogens in an emulsion of 500 μL of 0.15 M sodium phosphate and 500 μL of Freund's complete adjuvant (Difco, Detroit, Michigan). Four different immunizations were done depending on the DGL immunogen used (Figure 1) by injection of: (1) native DGL-G3-TFA salt at days 0, 21, 36, and 96 (Batch 1), (2) fluorescein-DGL-G3 at days 0, 21, and 36 (Batch 2), (3) DGL-G3-Histamine at days 0, 21, 36, and 96 (Batch 3), and (4) BSA-histamine at day 0, followed by injection of DGL-G3-histamine at days 21, 36, and 96 (Batch 4).

Sera collected 15 days after immunization (days 21, 36, and 96) were separated after blood coagulation by centrifugation at 2500 rpm for 10 min and stocked at −80 °C. All immunizations were done in a certified breeding center (agreement number B-34-172-23) in compliance with the local ethics committee.

Antibody Production and Characterization. Two tests were used: an indirect ELISA test to analyze antibody production and a competitive ELISA test to check the affinity of antibodies against the histamine hapten.

Indirect ELISA was performed on 96-well plates (NUNC, Maxisorp; Pierce Interchim, Montluçon, France). The well-plates were filled at 4 °C with 50 μL per well of immunoconjugate (DGL-G3, fluorescein-DGL-G3, DGL-G3-histamine, or BSA-histamine) or carrier (DGL-G3 or BSA) at 10 μg mL^{-1} in 10^{-2} M phosphate buffered saline (PBS) and allowed to stand overnight under orbital shaking. Wells were then rinsed twice with PBS and saturated for 2 h at 37 °C with 50 μL of 5% silk milk in PBS. Next, wells were rinsed twice with PBS and filled at 2 h at 37 °C with 50 μL of serum serially diluted (1/100 to 1/512 000) in 0.5% silk milk PBS buffer. Well plates were then washed with PBS and filled with 50 μL of 0.4 μg mL^{-1} sheep antirabbit IgG peroxidase diluted in PBS. After 1 h at 37 °C, wells were washed twice with PBS, and the color reaction was initiated by the addition of 200 μL of a 33% OPD solution. The colorimetric reaction was stopped after incubation for 15 min in the dark by the addition of 25 μL of a 4 N H_2SO_4 solution per well. The optical density (OD) was measured at 492 nm on an ELISA plate reader (Labsystems Multiskan Ex, Thermo Electron Corporation France). The blank control consisted of reactions made on noncoated wells. The B value corresponds to the OD value of the sample corrected by subtraction of the blank.

Competitive ELISA was performed as previously described.[4,5] In brief, after a 10 min Covalink well plates (NUNC, Merck Eurolab, France) preactivation with 200 μL of glutaraldehyde buffer (5% in sodium acetate 3 M), 200 μL of derivatized histamine (26 μg mL^{-1} dissolved in sodium acetate 3 M) was covalently linked to wells for 10 min and then washed three times with PBS Tween 20 (0.05%) buffer. Sera diluted at 1/64 000 were preincubated for 2 h at 37 °C with diluted immunoconjugates (concentration from 10^{-1} to 10^{-8} M) in PBS Tween 20 (0.05%). Then, 200 μL of each competitive sample was applied to wells previously coated with histamine derivatives. After 2 h at 37 °C, wells were washed three times with PBS Tween and filled with 200 μL of 1/10 000 sheep antirabbit IgG peroxidase diluted in PBS. Measurements using OPD were performed as described for indirect ELISA. Blank control consisted of reactions made on noncoated wells. Negative control consisted of well plates filled only with sheep antirabbit IgG peroxidase. Positive control consisted of well plates filled with diluted sera without competitors. B values were the OD values of the samples corrected by subtraction of blank. B0 value corresponds to the maximum OD that could be obtained with the positive control. Results are expressed using the B/B0 ratio.

Results and Discussion

Dendrigraft Poly-L-lysine Synthesis. Dendrigraft poly-L-lysine compounds (DGL) were synthesized by an iterative process in water at 0 °C, pH 6.5 (Scheme 2).[38] The first step of this synthesis consisted in the polymerization of the N$^{\varepsilon}$-TFA-lysine *N*-carboxyanhydride (N$^{\varepsilon}$-TFA-Lys-NCA) without any initiator leading to an insoluble product. Then, the insoluble product was collected, and the N$^{\varepsilon}$-TFA protecting group was removed (step 2), leading to the first generation of soluble DGL (DGL-G1). Step 3 was the reproduction of step 1, using DGL-G1 as the initiator. The TFA protecting group was removed (step 4) to obtain the second generation of soluble DGL (DGL-G2). Next generations of DGL (G3 to G5) were obtained using the same method. The precipitation of the insoluble compounds during the synthesis of DGL-Gn was under thermodynamic and kinetic control. This method ensures the reproducibility in mass and structure of these compounds made of biogenic building blocks only.[39] Moreover, by using this synthetic route, the polydispersity index, calculated using the weight-average MW divided by the number-average MW determined by light scattering measurements performed with each generation, was lower than 1.3.[43]

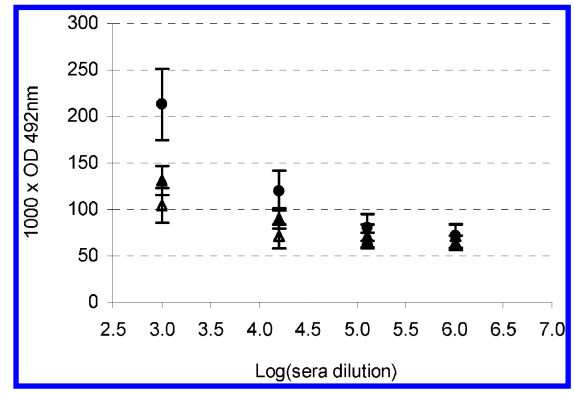


Figure 2. Antibodies concentration in rabbits' sera after two injections (day 36) of native DGL-G3-TFA salt. Prebled anti-DGL-G3 antibodies (●), anti DGL-G3 TFA salt antibodies (▲), and anti BSA antibodies (△). Each value reported here is a mean of two sera measures. Relative standard deviations are between 10 and 18%.

DGL-Gn synthesized here are fully soluble in water. They turned out to be nontoxic.[38] They are only composed of lysine residues on their periphery and have a high number of amine groups on their surface,[47] properties that are perfectly adapted for a use as antigen carriers. To assess the immunological properties of DGL-Gn, we focused on DGL third generation (DGL-G3), which has an MW of 22 kDa and present a mean number of 123 lysine residues on its surface. We studied first the ability of its native and functional forms to induce the production of antibodies in rabbits. Finally, we studied the properties of a combination of DGL-G3 and natural carrier to raise antibodies directed against the hapten.

DGLs As Furtive Carriers. At first, no antibodies were found against native DGL-G3-TFA salt (batch 1) in serum of rabbits immunized with DGL-G3-TFA salt (Figure 2). Moreover, the detection of antibodies directed against BSA or directed against DGL-G3-TFA salt showed identical results (Figure 2), and the OD background of prebled sample was higher than the sample from immunization. This result is in agreement with the known immunological properties of dendritic molecules, especially lysine dendrons,[21,48] and is in agreement with the hypothesis that protein with long disordered domains (longer than 30 residues) are less likely to be immunogenic.[49,50] Indeed, recent Raman chiroptical analysis suggests a predominant disordered structure in DGL-G3.[47]

At last, no antibodies directed against fluorescein-DGL-G3 (batch 2) were detected in the serum of rabbits after immunization with fluorescein-DGL-G3 (Figure 3). However, the fluorescein moiety is highly immunogenic. (At least 14 antifluorescein antibody products are commercially available up to now.) Therefore, our results suggest that fluorescein groups are either covered by lysine residues or not abundant enough on DGL to induce immune response (Figure 3). Similarly, antibodies directed against DGL-G3-histamine (batch 3) were not detected in the serum of rabbits immunized with DGL-G3-histamine (Figure 3).

In summary, DGL-G3 TFA salt, fluorescein-DGL-G3, and DGL-G3-histamine were not able to induce antibody production in rabbits', if injected alone during the immunization process.

Antibody Production with DGL-G3-Histamine. Rabbits were immunized by one injection of BSA-histamine, followed by two injections of DGL-G3-histamine (batch 4). The titer of antibodies directed against BSA, BSA-histamine, and DGL-G3-histamine was evaluated by direct ELISA analysis.

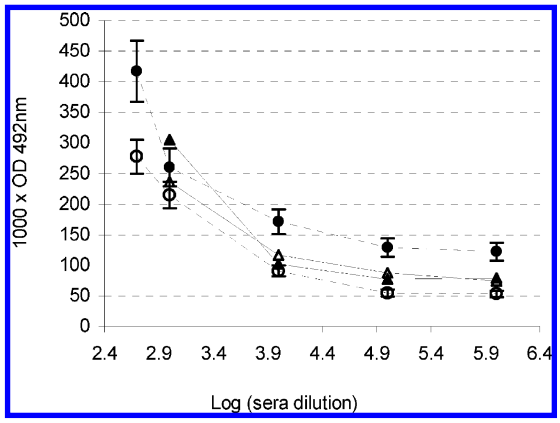


Figure 3. Antibody concentrations in sera of rabbits immunized with fluorescein-DGL-G3 (---) or DGL-G3-histamine (—). The measurements were made for anti-fluorescein-DGL-G3 antibodies prebled (●) and at day 36 (○), anti-DGL-G3-histamine antibodies at day 36 (▲), and anti DGL-G3 TFA salt antibodies (△).

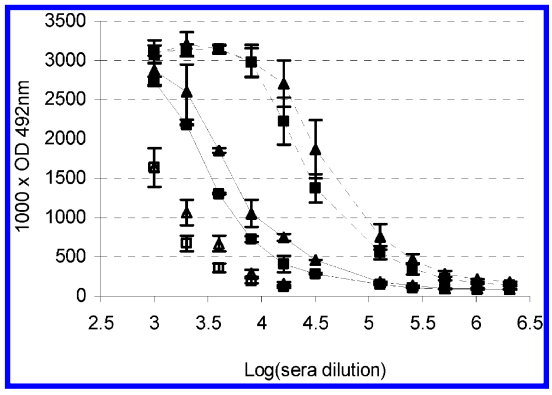


Figure 4. Antibody concentrations in rabbits' sera after immunization with BSA-histamine (■), followed by immunization with DGL-G3-histamine (▲). Determination for anti-BSA-histamine antibodies (---), anti-DGL-G3-histamine antibodies (—), and anti-BSA antibodies after first immunization (□) or second immunization (△).

Table 1. Titer of DGL-G3-Histamine and BSA-Histamine Antibodies in Rabbits' Sera

collecting days after the first immunizations	BSA-histamine antibodies titer	DGL-G3-histamine antibodies titer	ratio
21[a]	27695	3879	7.14
36	39994	5744	6.96
96	75647	38875	1.95

[a] Day of the 2nd immunization with DGL-G3-histamine. (For details, see the Experimental Section.)

Rabbits produced antibodies directed against BSA and BSA-histamine (Figure 4) 21 days after the first immunization with BSA-histamine. Those results are in agreement with all literature data dealing with successful antibody production as well as with previous results on raising antibodies directed against conjugated haptens.[3−5,44−46] Antibody titer, determined by ELISA, was higher when using BSA-histamine than when using DGL-G3-histamine (Figure 4, Table 1). At first, this could be explained by the structure of the DGL-G3, which presents at its periphery a mixture of lysine ε-NH_2 and α-NH_2 groups. Histamine linked to α-NH_2 lysine of DGL-G3 could not be as well recognized by antibodies as those linked to ε-NH_2 lysine of BSA. Second, antibodies directed against the natural BSA were produced and detected (Figure 4), whereas antibodies against DGL-G3 TFA salts were not detected.

A second injection of DGL-G3-histamine led to an increase in antibody titer measured against both BSA-histamine and DGL-G3-histamine (Table 1). This means that even if nonimmunogenic by itself, the DGL-G3-histamine was able to enhance the immune response against histamine. Moreover, a third immunization with DGL-G3-histamine increased the antibody specificity for conjugated histamine because the BSA-histamine/DGL-G3 histamine titer ratios decreased (Table 1). Indeed, the antibody affinity constants (K_m) after 1, 2, and 3 boosts for histamine at day 96 (Figure 5) were (8.6, 7.9, and 3.1) $\times$ 10^{-4} M, respectively. These results show that using DGL-G3-histamine as a carrier during the second immunization led to an increase in the affinity of antibodies directed against histamine. This could be attributed to a positive effect of the DGL-G3-histamine carrier despite its furtive nature when used alone. These results are in agreement with our previous data on antibody production from conjugated haptens using three different carriers including linear poly lysine.[3−5]

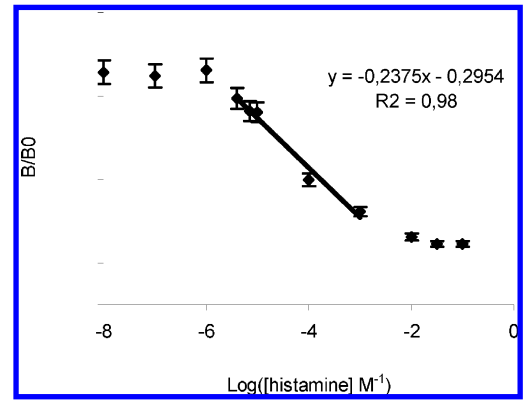


Figure 5. Competition results at day 96. The linear regression equation was calculated with five points for the affinity constant determination.

Conclusions

The third generation of native dendrigraft poly-L-lysine (DGL-G3) is nonimmunogenic by itself. Used as second carrier, DGL-G3 is very useful for antibody production and, to be more precise, increases the specificity of antibody against conjugated haptens. DGL-G3 has an ideal MW of 22 kDa, is nontoxic, and its production can be scale up. DGL-G3 appears consequently to be a good carrier able to improve the specificity of antibodies for diagnostic immunoassays. By extension, DGL-G3 might also be a powerful carrier candidate for vaccine production.

Acknowledgment. We thank the French National Space agency for financial support.

References and Notes

(1) Tam, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 5409–5413.
(2) Sadler, K.; Tam, J. P. *Rev. Mol. Biotechnol.* **2002**, *90*, 195–229.
(3) Vandenabeele-Trambouze, O.; Geffard, M.; Bodet, D.; Despois, D.; Dobrijevic, M.; Grenier-Loustalot, M.-F.; Commeyras, A. *Chirality* **2002**, *14*, 519–526.
(4) Claeys-Bruno, M.; Vandenabeele-Trambouze, O.; Sergent, M.; Geffard, M.; Bodet, D.; Dobrijevic, M.; Commeyras, A.; Phan-Tan-Luu, R. *Chemom. Intell. Lab. Syst.* **2006**, *80*, 186–197.
(5) Claeys-Bruno, M.; Vandenabeele-Trambouze, O.; Sergent, M.; Geffard, M.; Bodet, D.; Dobrijevic, M.; Commeyras, A.; Phan-Tan-Luu, R. *Chemom. Intell. Lab. Syst.* **2006**, *80*, 176–185.
(6) Mangas, A. R.; Coveñas, R.; Geffard, K.; Geffard, M.; Marcos, P.; Insausti, R.; Dabadie, M. P. *Neurosci. Lett.* **2004**, *79*, 258–261.
(7) Mangas, A. R.; Coveñas, R.; Geffard, K.; Geffard, M.; Marcos, P.; Insausti, R.; Dabadie, M. P. *Life Sci.* **2006**, *79*, 1121–1128.

(8) Mangas, A. R.; Coveñas, R.; Bodet, D.; Geffard, M.; Aguilar, L. A.; Yajeya, J. *Neuroscience (Oxford)* **2007**, 144.
(9) Wang, N.-D.; Chen, J.-H.; Zhang, X.; Chen, L.-M.; Jin, S. *Huaxue Xuebao* **2001**, *59*, 1798–1802.
(10) Guo, J.; Liu, Y.; Gao, Z.; Zhang, W.; Wu, Q.; Guo, Y. *Zhongguo Gonggong* **2003**, *19*, 1069–1071.
(11) Gui, W.; Zhu, G.; Wang, S.; Guo, Y.; Jin, R. *Faming Zhuanli Shenqing Gongkai Shuomingshu* **2007**, CN 1908664 A 200770207.
(12) Liu, Y.; Zhong, Q.; Lei, H.; Zhang, T.; Yu, H.; Sun, Y. *Weisheng Yanjiu* **2008**, *37*, 234–236.
(13) Yang, Z.; Gao, J.; Situ, W.; Zhang, Q.; Li, F. "Method for producing anti-hapten antibodies by using serum albumins from homogeneous animals as carrier proteins" from Faming Zhuanli Shenqing Gongkai Shuomingshu (2008), patent number CN 101139397 A 20080312.
(14) Sakarellos-Daitsiotis, M.; Krikorian, D.; Panou-Pomonis, E.; Sakarellos, C. *Curr. Top. Med. Chem.* **2006**, *6*, 1715–1735.
(15) Freche, J. P. Strategy of Immunogen Production. In *Methods Immunological Analysis: Samples and Reagents*; Masseyeff, R. F., Albert, W. H., Staines, N. A., Eds.; VCH Publishers: New York, 1993; Vol. 2, pp 54−65.
(16) Rajnavolgyi, E.; Lanyi, A.; Hudecz, F.; Kurucz, I.; Kiss, K.; Laszlo, G.; Szekerke, M.; Gergely, J. *Mol. Immunol.* **1989**, *26*, 949–958.
(17) Trudelle, Y.; Brack, A.; Delmas, A.; Pedoussaut, S.; Rivaille, P. *Int. J. Pept. Protein Res.* **1987**, *30*, 54–60.
(18) Gokulan, K.; Khare, S.; Rao, D. N. *DNA Cell Biol.* **1999**, *18*, 623–630.
(19) Siemion, I. Z.; Kluczyk, A. *Peptides* **1999**, 20.
(20) Green, L. S.; Bell, C.; Janjic, N. *BioTechniques* **2001**, *30*, 1094–1096.
(21) Roberts, J. C.; Bhalgat, M. K.; Zera, R. T. *J. Biomed. Mater. Res.* **1996**, *30*, 53–65.
(22) Tam, J. P.; Lu, Y.-A.; Yang, J.-L. *Eur. J. Biochem.* **2002**, *269*, 923–932.
(23) Riley, E. M.; Young, S. C.; Secombes, C. J. *Vet. Immunol. Immunopathol.* **1996**, *55*, 243–253.
(24) Culshaw, S.; LaRosa, K.; Tolani, H.; Han, X.; Eastcott, J. W.; Smith, D. J.; Taubman, M. A. *Infect. Immun.* **2007**, *75*, 915–923.
(25) Argiro, L.; Henri, S.; Dessein, H.; Kouriba, B.; Dessein, A. J.; Bourgois, A. *Vaccine* **2000**, *18*, 2033–2038.
(26) Haro, I.; pérez, S.; Garcia, M.; Chanb, W. C.; Ercillac, G. *FEBS Lett.* **2003**, *540*, 133–140.
(27) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149–2154.
(28) Basak, A.; Boudreault, A.; Chen, A.; Chretien, M.; Seidah, N.; Lazure, C. *J. Pept. Sci.* **1995**, *1*, 385–395.
(29) Keah, H.; Kacorius, E.; Hearn, M. *J. Pept. Res.* **1998**, *51*, 2–8.
(30) Tam, J. P.; Xu, J.; Eom, K. D. *Biopolymers* **2001**, *60*, 194–205.
(31) Tam, J. P.; Spetzler, J. C. *Biomed. Pept., Proteins Nucleic Acids* **1995**, *1*, 123–132.
(32) Tam, J. P. PCT Int. Appl. WO9500846, 1995.
(33) Tam, J. P.; Yu, Q.; Lu, Y.-A. *Biologicals* **2001**, *29*, 189–196.
(34) Tam, J. P.; Yu, Q.; Yang, J.-L. *J. Am. Chem. Soc.* **2001**, *123*, 2487–2494.
(35) Li, X.; Zhang, L.; Hall, S. E.; Tam, J. P. *Tertrahedron. Lett.* **2000**, *41*, 4069–4073.
(36) Kalia, J.; Raines, R. T. *Angew. Chem., Int. Ed.* **2008**, *47*, 7523–7526.
(37) Dirksen, A.; Hackeng, T. M.; Daxson, P. E. *Angew. Chem., Int. Ed.* **2006**, *45*, 7581–7584.
(38) Commeyras, A.; Collet, H.; Souaid, E.; Cottet, H.; Romestang, B.; Vandenabeele-Trambouze, O. INPI PCT/FR2006/000952, 2006.
(39) Collet, H.; Souaid, E.; Cottet, H.; Deratani, H.; Boiteau, L.; Dessalces, G.; Rossi, J.-C.; Commeyras, A.; Pascal, R. *Chem.—Eur. J.* **2010**, *16*, 2309–2316.
(40) Tomalia, D. A.; Frechet, J. M. J. *J. Polym. Sci., Part A: Polym. Chem.* **2002**, *40*, 2719–2728.
(41) Teertstra, S. J.; Gauthier, M. *Prog. Polym. Sci.* **2004**, *29*, 277–327.
(42) Gauthier, M. *J. Polym. Sci., Part A: Polym. Chem.* **2007**, *45*, 3803–3810.
(43) Cottet, H.; Martin, M.; Papillaud, A.; Souaïd, E.; Collet, H.; Commeyras, A. *Biomacromolecules* **2007**, *8*, 3235–3243.
(44) Geffard, M.; Dulluc, J.; Heinrich-Rock, A. M. *J. Neurochem.* **1985**, *44*, 1221–1228.
(45) Geffard, M.; Seguela, P.; Heinrich-Rock, A. M. *Mol. Immunol.* **1984**, *21*, 515–522.
(46) Chagnaud, J. L.; Mons, N.; Tuffet, S.; Grandier-Vazeilles, X.; Geffard, M. *J. Neurochem.* **1987**, *49*, 487–494.
(47) Coussot, G.; Nicol, E.; Commeyras, A.; Desvignes, I.; Pascal, R.; Vandenabeele-Trambouze, O. *Polym. Int.* **2009**, *58*, 511–518.
(48) Green, I.; Paul, W. E.; Benacerraf, B. *J. Exp. Med.* **1966**, *123*, 859–879.
(49) Xie, H.; Vucetic, S.; Lakoucheva, L. M.; Oldfield, C. J.; Dunker, A. K.; Uversky, V. N.; Obradovich, Z. J. *Proteom Res.* **2007**, *6*, 1882–1898.
(50) Uversky, V. N.; Oldfield, C. J.; Dunker, A. K. *J. Mol. Recognit.* **2005**, *18*, 343–384.

BM9012056