

Dendrimerized Cellulose as a Scaffold for Artificial Antigens with Applications in Drug Allergy Diagnosis

Maria I. Montañez,[†] Ezequiel Perez-Inestrosa,^{*,†} Rafael Suau,[†] Cristobalina Mayorga,[‡]
Maria J. Torres,[‡] and Miguel Blanca[‡]

*Department of Organic Chemistry, Faculty of Sciences, University of Malaga, 29071 Malaga, Spain,
and Research Unit for Allergic Diseases, "Carlos Haya" Hospital, 29010 Malaga, Spain*

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Cellulose-based dendrimerized material was prepared and its quality was assessed by determining the number of amine functional groups incorporated. Based on the results for a series of preparations, the material was obtained in a highly reproducible manner thanks to the particular chemical construction method used. The number of amine groups incorporated and the amount of dendrimer attached are directly related to the dendrimer generation. The combination of the properties of the cellulose polymer and those of the dendrimeric state provides biocompatible materials amenable to easy chemical characterization. The proposed method provides an effective tool for developing clinically testable materials with a view to studying adverse immunological responses to drugs in humans.

Introduction

Polymers containing a plurality of free hydroxyl groups (e.g., cellulose) are effective solid substrates for immobilizing biomolecules with a view to developing biosensors and immunoassay kits.¹

Cellulosic materials have been widely used to immobilize biomaterials on account of their accessibility, hydrophilicity, low cost, and large number of surface hydroxyl groups amenable to chemical reaction. Cellulose has been subjected to a vast variety of alterations to prepare new derivatives with various supramolecular architectures for use in a broad range of applications, including ultrathin coatings,^{2–4} host–guest systems,^{5–7} biosensors,^{8–10} and biomaterials.^{11–14}

Developing effective new materials and better methodological procedures for solid-phase assays requires improving the flexibility and the analytical selectivity and sensitivity of existing choices. These constructs are referred to as “architectural copolymers”, as first described by Tomalia et al.^{15,16} By combining the linear polydisperse homopolymer properties of cellulose with the chemistry and topology of the dendritic state,^{17–19} one can exploit the advantages of these spherical, highly branched macromolecules to provide benefits for industrial applications on solid supports, where ultimate perfection in structural uniformity is not essential, and obtaining new, flexible materials should be more accessible.

The binding of an immunoreactive component such as an analyte-specific antigen to a specific antibody immobilized on a solid-phase support is the essential, common feature of solid-phase immunoassay techniques. Such techniques are used to measure circulating levels of a number of markers used as guidance in the management of patients with specific clinical symptoms. For example, the RadioAllergoSorbent test (RAST) is a blood test used to determine what a person is allergic to based on the amount of immunoglobulin E (IgE) reacting specifically with suspected or known allergens.²⁰ IgE is the antibody associated with an allergic response: if a person

exhibits a high level of IgE directed against an antigen, then the test may indicate that the person in question is allergic to it. These tests have some advantages, including increased sensitivity without loss of specificity and excellent reproducibility throughout the measuring range of the calibration curve. Also, blood (in vitro) testing has many advantages over skin-prick (in vivo) testing; thus, the patient's medication need not always be suspended, the results are not affected by the patient's skin conditions, and the method is less invasive and less prone to introducing potentially hazardous substances into the patient's body.

β -Lactam antibiotics constitute the drug class most frequently administered against IgE-mediated allergy, which occurs in up to 10% of patients treated with high penicillin doses.²¹ We recently²² synthesized and characterized a series of densely penicilloylated poly(amidoamine) (PAMAM) dendrimers from generations 0 to 2 (GnBPO, $n = 0–2$) with a precisely defined chemical structure and conducted preliminary RAST inhibition tests in solution by using sera from patients with an immediate allergic reaction to penicillin, the patients who exhibited disparate RAST levels showing that recognition by IgE antibodies directed to benzylpenicillin occurred to an extent increasing with increasing dendrimer size (i.e., with increasing hapten density). These functionalized dendrimers allowed us to conclude that hapten-carrier (dendrimer) conjugates in solution mimic the recognition of natural hapten-carrier (protein) conjugates. The structural precision of dendrimers has prompted a number of studies aimed at developing new biochemical applications such as the amplification of molecular effects or the production of extremely high local concentrations of drugs, molecular labels, or probe moieties.²³ However, their potential for emulating the carrier protein in conjugates for IgE recognition has not yet been explored. On the belief that PAMAM dendrimers, based on their shape and physical properties,²⁴ can be viewed as protein mimetics and have potential as protein-like materials for biotechnological applications, we prepared penicilloylated dendrimers from generations 0–6 on cellulose surfaces as platforms for detecting IgE antibodies from penicillin-allergic patients.

* To whom correspondence should be addressed. E-mail: inestrosa@uma.es.

[†] University of Malaga.

[‡] “Carlos Haya” Hospital.

This paper reports recent progress in the production of a series of cellulose solid phases chemically functionalized with densely penicilloylated PAMAM dendrimers (dendrimer generation **n** benzylpenicilloyl; **DGnBPO**, $n = 0-6$) of increasing binding epitope density and size scaling. We studied also the use and evaluation of this panel of dendrimers in the recognition and quantification of IgE antibodies directed to benzylpenicillin and the effects of changes in ligand valency related to changes in epitope presentation (viz. residue density and architecture).

Experimental Section

Materials. Poly(amidoamine) dendrimers (ethylenediamine core) with amino surface groups (PAMAM generations 0–6) were purchased from Dendritic Nanotechnologies, Inc. (dnt) as solutions in methyl alcohol. Benzylpenicillin sodium salt was supplied by CEPAC, and S.L. Standard chemicals were obtained from Aldrich or Merck and used without further purification. Cellulose discs 6 mm in diameter were prepared from Whatman 54 filter paper (hardened circles Ø 185 mm). Phosphate buffer saline (PBS) was prepared as described elsewhere.²⁵

Activation of the Cellulose Solid Phase with Cyanogen Bromide.

Preparation of C-OCN Discs. A number of discs weighing approximately 1 g (in the region of 50) was washed with distilled water for 30 min during which the washing medium was changed four times. Then, the washing medium was removed, the discs were immersed in water, and the pH was adjusted to 11 with 2 N NaOH. Next, a solution of BrCN (1 g) dissolved in the minimum volume of acetone was added to each 1 g of discs under shaking and pH control for 1 h. The 2 N NaOH solution was used to prevent the pH from falling below 10.5. Addition of the base was stopped as soon as the pH leveled off at 11. After treatment, the discs were washed with aqueous 0.1 M NaHCO₃ five times, followed by a 40 mL volume of mixtures of water with 25, 50, and 75% acetone (once, once, and twice, respectively), and, finally, pure acetone twice. Then, the discs were dried in a desiccator under vacuum for 12 h and stored in freezer until needed.

Covalently Attaching PAMAM Dendrimers (G0–G6) to Activated Cellulose. Groups of 50 discs were placed into individual vials and supplied with 2 mL of a solution of PAMAM dendrimer (Gn 0–6) at a final concentration of 10 mg/mL in 0.1 M NaHCO₃, and the resulting suspension was shaken at room temperature for 4 h. The solution was then removed and the discs were washed with a 0.1 M solution of NaHCO₃ four times. Those discs to be subjected to the ninhydrin test were washed with water, filtered off, and dried; all others were ready for subsequent reaction.

Neutralizing Unreacted Activated Hydroxyl Groups. *Preparation of C-DGn Discs.* Groups of 50 discs were treated with a solution of 50 mM ethanolamine in 0.1 M NaHCO₃ for 1 h. Then, the solution was decanted and the discs were washed with an aqueous solution of 0.1 M NaHCO₃ three times for 30 min, followed by 0.05 M Na₂CO₃/NaHCO₃ buffer at pH 10.2. The discs to be used as blanks in the RAST (carrier without hapten) were stored in 0.05 M Na₂CO₃/NaHCO₃ buffer at pH 10.2 at 5 °C until analysis, whereas all others were ready for subsequent reaction.

Covalently Binding Penicillin G to PAMAM Dendrimer Surfaces.

Preparation of C-DGnBPO Discs. Groups of 50 discs were treated with 2 mL of a freshly made solution of penicillin G (10 mg/mL) in 0.05 M Na₂CO₃/NaHCO₃ aqueous buffer at pH 10.2 at 4 °C with gentle agitation for 6 days, with 1 mL of buffer being added to the suspension at approximately 24 h intervals. Then, the solution was decanted and the discs were washed with 0.1 M NaHCO₃ twice, followed by alternate washing with 0.1 M NaHCO₃ and 0.1 M acetate buffer. Finally, the discs were washed with PBS three times and stored in PBS at 4 °C for later use (RAST assay or ninhydrin test).

Quantitation of Free Primary Amine Groups Bonded to the Solid Phase. The amine test solution consisted of ninhydrin (100 mg) dissolved in 95% EtOH (10 mL). The amine test was previously applied

to 10 µL of a 2 mM solution of butylamine in water, as a standard solution. The absorbance of the Ruhemann's purple complex at 576 nm in EtOH was measured: Abs (576 nm) = 0.264; $\epsilon = 26416 \text{ M}^{-1} \text{ cm}^{-1}$. Then, a disk of functionalized cellulose was suspended in a 2 mL aliquot of the ninhydrin test solution in a test tube and heated under reflux for 45 min, the resulting suspension then being allowed to cool. Next, the reaction volume was made to 2 mL with 95% EtOH and the solution absorption was measured at 570 nm, with the number of amine groups present per milligram of cellulose being calculated from ϵ .

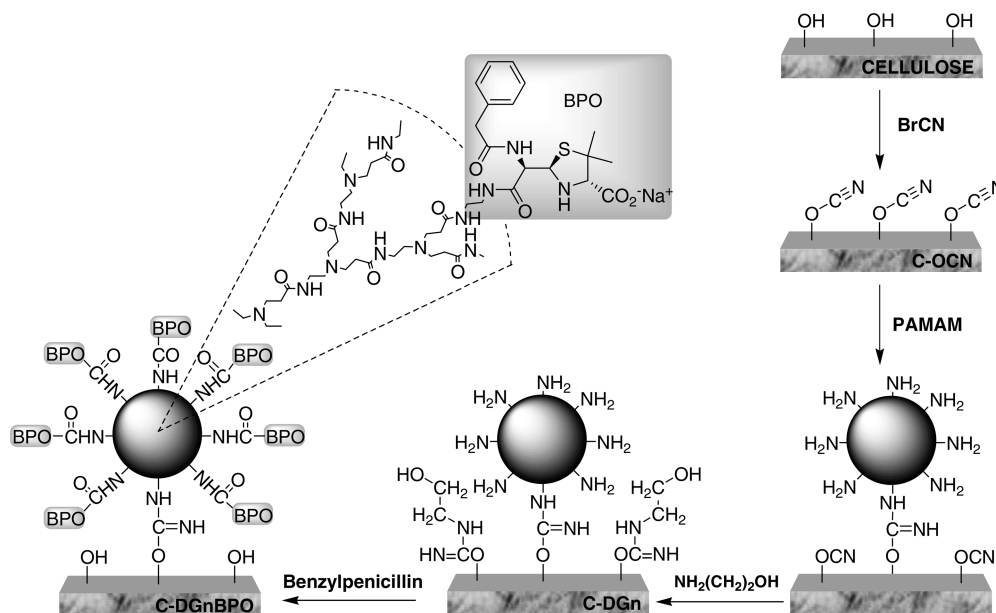
Selection of Cases and Radioimmunoassay for IgE Determination. The patients studied had been diagnosed with an immediate allergic reaction to penicillins by following a well-validated protocol (ENDA).²⁶ RAST to benzylpenicillin was performed as described elsewhere.²⁵ Briefly, a 30 µL volume of patient serum was incubated with dendrimerized cellulose discs containing various penicillin–dendrimer conjugates for 3 h. After three washes, radiolabeled anti-IgE antibody was added and the solutions were allowed to incubate overnight. The discs were then washed and their radioactivity was measured in a Cobra II autogamma gamma counter. The results were calculated as percentages of the maximum possible value (% RAST).

Results

Preparation of Cellulose Discs. The cellulose discs used as solid supports served as scaffolds for anchoring the **DGnBPO** penicilloylated dendrimers.²⁷ Biologically active proteins and polypeptides can couple in various ways to cellulose. The use of cyanogen halides for this purpose ensures a high yield of bound polypeptide or protein retaining a substantial portion of its activity.²⁸ As shown in Scheme 1, cellulose surfaces were activated (**C-OCN**) by converting hydroxyl groups into cyanate groups by reaction with cyanogen bromide first and amino groups in the PAMAM dendrimers ($G = 0-6$) reacted then to obtain the isourea derivative through which the dendrimer anchored to the solid surface. The intact cyanate groups remaining were deactivated by reaction with 2-aminoethanol to obtain the solid surface supported dendrimers (**C-DGn**). This procedure provides a chemically modified surface onto which dendrimers can be covalently attached with a view to facilitating the covalent immobilization of a variety of clinically significant analytes. In this work, dendrimerized surfaces were penicilloylated (**C-DGnBPO**) to obtain the “major” antigenic determinant (BPO) directed to benzylpenicillin.

Characterization of Dendrimerized Cellulose Discs. To precisely assess the ability of the cellulose discs to support the artificial dendrimerized antigens to be used for serum IgE detection, we estimated the total amount of dendrimers of each generation supported on the cellulose discs, **C-DGn**, and the total amount of BPO groups covalently bonding to surface amine groups per dendrimer to form **C-DGnBPO**. The number of free primary amine groups present in **C-DGn** is a good measure of the amount of PAMAM dendrimers covalently bonded to activated cellulose discs. Such a number was determined by reacting the dendrimerized cellulose discs with ninhydrin and monitoring the resulting brightly colored deep blue or purple compound (Ruhemann's purple) in quantitative terms.²⁹ Because ninhydrin development of this color is selective for ammonia or primary amines without interference of secondary or tertiary amines, or amine groups next to a tertiary carbon that cannot be detected with the ninhydrin test,³⁰ the method enabled the selective quantitation of surface primary amine groups in the dendrimers immobilized on the cellulose discs.

By way of example, Figure 1 shows the results of the determination of the number of free amine groups per gram of cellulose disk **C-DG2** obtained by coupling PAMAM generation 2 to activated cellulose discs. The graph shows the results for

Scheme 1. Modification of Cellulose Surfaces To Generate Dendritic-Linked Systems for the Preparation of Solid Surface Supported GnBPO Conjugates

five different samples and reflects the reproducibility of the dendrimer coupling and functionalization methods. Tests were conducted on all seven generations studied (see Supporting Information).

For better understanding and easier comparison of the ability of each dendrimer generation to bind to the cellulose discs for subsequent penicilloylation, the results are also shown in Table 1. The second column shows the average number of amine groups contained in each group of **C-DGn** dendrimerized cellulose discs per gram of cellulose as determined in 10 runs (individual values are given in the Supporting Information). The fact that the ninhydrin test revealed the presence of amine groups confirmed that dendrimers are attached to activated cellulose to form discs such as **C-DGn**. The depiction of dendrimers as spherical structures is assumed only in solution. On a surface, studies have described more distorted structures of dendrimers and that the dendrimers flatten on the surface.^{31–33} In this way, at least one primary amine group at the dendrimer surface reacted with the cyanate derivative present in activated cellulose, the remaining amine groups being available for chemical reaction. The number of free amine groups was found to be

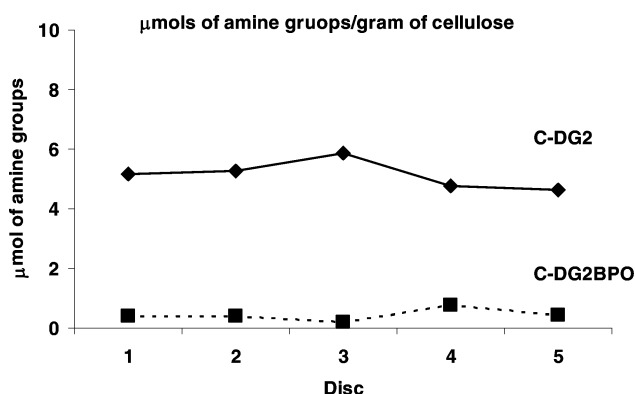
Table 1. Quantitation of the Amount of Dendrimers Attached to Cellulose Discs

generation <i>n</i> (amine groups)	μmol of amine groups/ gram of cellulose		μmol of dendrimer/ gram of cellulose
	C-DGn	C-DGnBPO	
0 (4)	2.2	1.7	0.724
1 (8)	3.8	0.9	0.543
2 (16)	5.3	0.4	0.354
3 (32)	4.1	0.8	0.133
4 (64)	3.4	0.3	0.053
5 (128)	1.6	0.6	0.013
6 (256)	0.7	0.4	0.003

generation-dependent, and a Gaussian-like distribution as a function of the dendrimer generation bonded to cellulose discs was obtained. Anchored dendrimers were penicilloylated to obtain **C-DGnBPO** in accordance with Scheme 1. This involved reacting free amine groups at the dendrimer surface with the carbonyl group in the β -lactam ring of benzylpenicillin. Free primary amine groups (third column in Table 1) were now quantified to check the ability of this chemical strategy to ensure binding of the BPO allergenic determinant to the surface of the anchored dendrimer to generate artificial dendrimerized antigens useful for IgE recognition. As can be seen by comparing the previous results with those in the first column, the number of primary amino groups at the dendrimer surface remaining free after the reaction with benzylpenicillin was very small. Consequently, the majority of these amino groups at the dendrimer surface have reacted with benzylpenicillin.

If we consider the number of primary amino groups per each generation of PAMAM dendrimer and that at least one of these amino groups are involved in the linkage to the cellulose disk, by comparing the data with those in the first column, one can estimate the amount (μmol) of dendrimers attaching to the cellulose discs per gram of cellulose. This is quite an important datum as it represents the number of artificial synthetic dendrimeric antigens that can be covalently supported on the cellulose discs and dictates the ability of these artificial materials to interact with IgE as a substrate.

RAST Results. Sera from 12 patients diagnosed with an immediate allergic reaction to benzylpenicillin were used to

**Figure 1.** Results of the determination of the total amount of primary amine bound to cellulose discs before (♦) and after (■) reaction with benzylpenicillin. The data correspond to five different samples of cellulose discs assayed with the ninhydrin method and are expressed in micromols of amine per gram of cellulose.

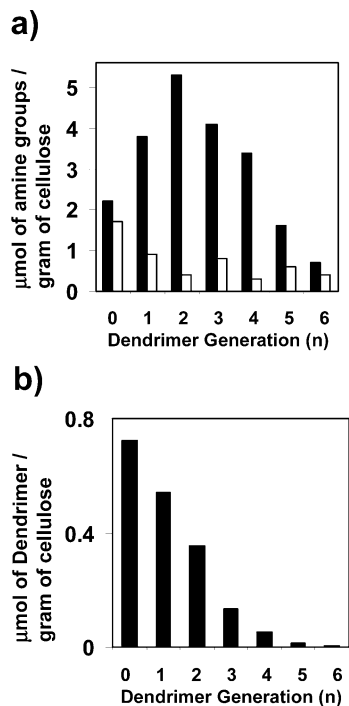


Figure 2. Results of the determination of free primary amino groups at the dendrimer surface attached to cellulose discs expressed in (a) μmol amine groups before (■) and after (□) reaction with benzylpenicillin and (b) μmol dendrimer.

check the usefulness of dendrimerized cellulose discs for detecting specific IgE antibodies directed to benzylpenicillin and compare the binding capacity of dendrimers from generations 0–6 used as carrier molecules (Figure 3). The ability of these materials to detect IgE antibodies was assessed via RAST tests; overall, the dendrimerized discs exhibited RAST levels. Both types of sera studied (viz. sera with low and high specific IgE levels) exhibited a certain RAST level with all dendrimer generations used; therefore, dendrimerized cellulose discs interact in an effective way to bind IgE antibodies. The penicilloylated dendrimers supported on the cellulose discs were recognized by the specific IgE antibodies because they mimicked the antigens toward which they were directed.

The results exhibited some dependence on the particular dendrimer generation attached to the discs and the corresponding RAST levels for each type of serum. As can be seen from Figure 3, the lower generations of dendrimer conjugates tended to exhibit the lower RAST binding levels. In fact, RAST level increases with increasing generation number and peaked around generation 3 or 4. However, the highest generations studied (5th and sixth) exhibited decreased or, less often, constant, RAST levels.

These results expose a decisive relationship between RAST level, the hapten-dendrimer conjugate generation ensuring adequate selectivity in the recognition of the IgE antibody and the topological characteristics of the dendrimerized cellulose discs.

Discussion

The development of new materials for biomedical applications is an expanding area of research that requires the implementation of new knowledge and technologies, but also the assimilation for the long-established medicinal area. For easy adaptation, the new, emerging techniques and materials must be validated

and tested on real samples and in the most representative universe of cases. The occurrence of selective allergic responses to β -lactam antibiotics, which constitute the most common type of adverse drug reactions mediated by specific immunological mechanisms, have raised increasing concern and, in response, the need to develop new, effective in vitro methods for detecting adverse drug reactions in clinical practice.

The robustness of an in vitro method to be applied in medicinal studies is determined not only by its sensitivity, selectivity, and reproducibility, but also by the procedure used to design the material; together, they can ensure optimum interaction with biological samples. Thus, sera, where immunoglobulins are present, are aqueous samples and the material to be applied has to lack hydrophobic character; also, they should be biocompatible to avoid spurious results in the form of false positives or negatives.

The amenability of the hydroxyl groups in cellulose to chemical manipulation allowed their conversion in a very high proportion into cyanate groups, which facilitated reaction with nucleophilic primary amine groups at the dendrimer surface to obtain cellulose anchored PAMAM dendrimers **C-DG_n** of different generations. In this way, we combined the properties of a straight chain homopolymer with the accuracy of the dendrimeric architecture. This allowed us to design polystructured solid supports facilitating the development of a comprehensive collection of building blocks of widely variable geometries and chemical functionalities.

The quality of the new material can be systematically controlled by determining the total amount of free amino groups present in the modified cellulose discs (**C-DG_n**). As can be seen from Figure 1, the proposed method is quite reproducible and provides materials with an identical degree of functionalization in each operation (i.e., homogeneous, reproducible materials). As can be inferred from the results of Table 1 and Figure 2a, the number of amino groups present in the cellulose discs is dependent on the particular dendrimer generation used. The number of amine groups does not vary in a linear manner, however; rather, it exhibits a Gaussian-like distribution centered on generation 2. Based on the total amount of surface amino groups present in each PAMAM dendrimer and of the fact that at least one anchored to the cellulose discs, we estimated the amount of dendrimer, in micromoles, attached to the discs per gram of cellulose (see table 1 and figure 2b). As can be seen, the number of dendrimers supported on the cellulose discs decreased with increasing dendrimer generation number. This can be ascribed to the ability of low generation dendrimers to migrate to inner locations in the cellulose disk tangle and to access by higher generations being restricted to the most outer locations. As a result, using a high dendrimer generation will result in partial anchorage of the dendrimer at “outer” positions, whereas using a low generation number will result in a more homogeneous distribution of dendrimers, which will reside in both “inner” and “outer” locations. Therefore, more reactive positions will be accessible to low generation dendrimers than to high generation dendrimers.

However, as can be inferred from Table 1 (see also Figure 1) by comparing the amount of amine groups, in micromoles, before and after benzylpenicillin conjugation the relatively small-sized molecule of benzylpenicillin can diffuse throughout the cellulosic material and react with free primary amine groups at the surface of anchored dendrimers. Thus, virtually all primary amino groups reacted to bind the BPO epitope to the dendrimer surface and produce **C-DG_nBPO**, and only a residual number of such groups remained unreacted.

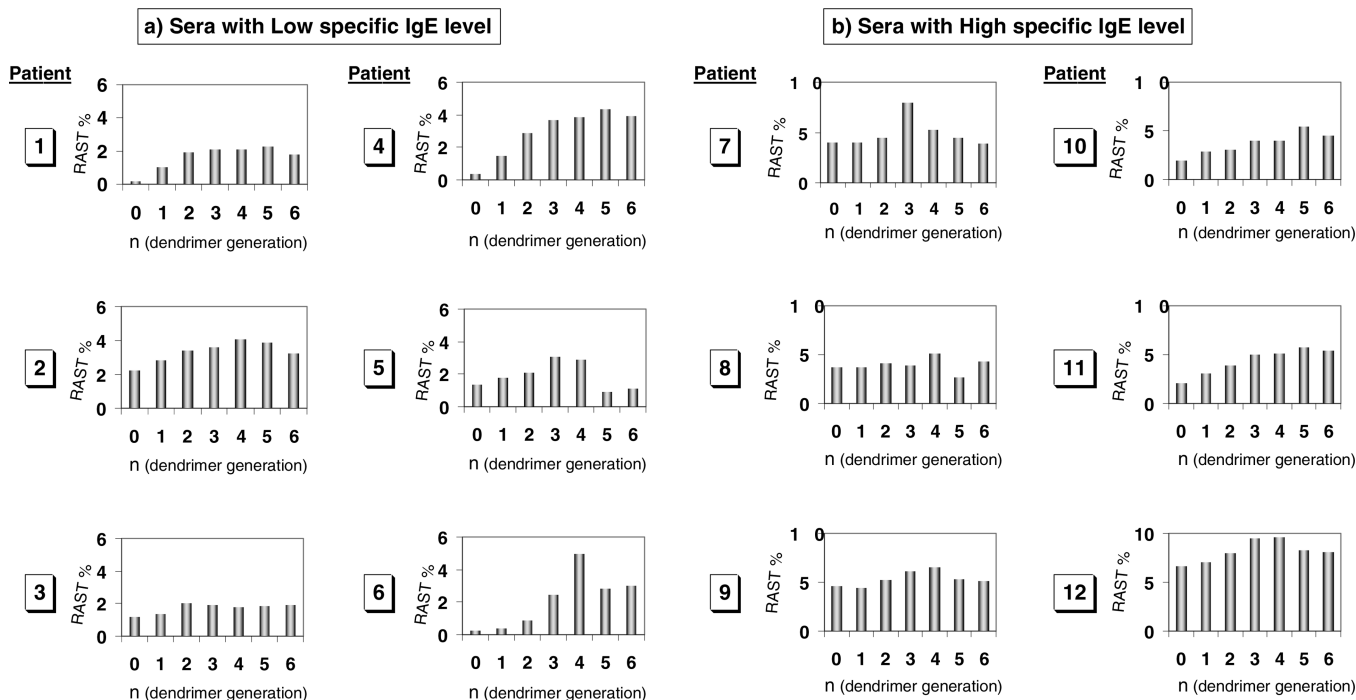


Figure 3. RAST values (as % RAST, y-axis) for patients' sera as a function of the generation number (n , x-axis) used to prepare the solid surface supported **DGNBPO** conjugates. Sera with (a) low and (b) high specific IgE levels were used. Samples were considered positive if they were higher than 2.5% of label uptake, which was the mean + 2SD of the negative control group.

The ability of the new dendrimeric materials to operate in biological samples was tested by RAST assay of sera from patients allergic to benzylpenicillin (Figure 3). The most immediate result was that, whichever the PAMAM generation used, a positive RAST was obtained. Therefore, the dendrimers anchored to the cellulose discs and subsequently functionalized at the surface with the “major” antigenic determinant directed to β -lactam antibiotics, produce a hybrid material (a cellulosic polymer combined with a dendrimeric architecture) that efficiently integrates the molecular requirements to be recognized by IgE antibodies. As a result, such antibodies are immobilized at cellulose disk surfaces and can be detected by radioimmunoassay. In this way, sera with IgE levels can be classified as positive (or negative) with a view to facilitating a patient's clinical diagnosis.

Sera with both low and high specific IgE levels can be detected with these dendrimerized material. This testifies to the usefulness of the new materials for developing new clinical tests. Based on Figure 3, RAST values are strongly dependent on the particular dendrimer generation used: discs coated with low generation dendrimers exhibit low RAST levels. Beyond the third generation, however, RAST levels fall with both types of serum.

Conclusion

As shown here, the properties of cellulose as a supporting platform can be integrated with the intrinsic structural properties of dendrimers to obtain new, flexible materials for studying biological samples. Cellulose hydroxyl groups were transformed into reactive cyanate groups and reacted with free primary amine groups at the dendrimer periphery to anchor PAMAM dendrimers from generations 0–6 to a cellulose disk structure. The quality of these materials (expressed as the total number of free primary amine groups present or as the amount, in micromoles, of dendrimers attached) can be assessed with the ninhydrin

method. The chemical methodology used in this work allows standardized materials with a definite total amount of attached dendrimers to be reproducibly prepared.

The new material has been successfully used to detect IgE in patients diagnosed with an immediate allergic reaction to benzylpenicillin. Although some fine-tuning will be required to establish an optimum diagnosis method, the RAST results obtained in this work suggest that dendrimerized cellulose discs meet all the requirements for developing clinically useful diagnostic tests.

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Supporting Information Available. Spectroscopic methodology and tables with individual determinations of the cellulose discs. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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*Maria I. Montañez,[†] Ezequiel Perez-Inestrosa,^{*,†} Rafael Suau,[†] Cristobalina Mayorga,[‡]
Maria J. Torres,[‡] and Miguel Blanca[‡]*

Department of Organic Chemistry, Faculty of Sciences, University of Malaga, 29071 Malaga, Spain and Research Unit for Allergic Diseases, “Carlos Haya” Hospital, 29010 Malaga, Spain.

E-mail: inestrosa@uma.es

[†] University of Malaga. [‡] “Carlos Haya” Hospital.

Quantitation of the amount of amine groups and dendrimers attached to cellulose discs: individual values for the seven generation of PAMAM dendrimers studied

Cellulose Disc		DG0		DG1	
X=		-NH ₂	PAMAM	-NH ₂	PAMAM
measures of different discs	1	1.6	0.534	2.0	0.280
	2	1.8	0.584	4.1	0.588
	3	2.1	0.708	4.7	0.677
	4	1.5	0.512	3.0	0.424
	5	1.8	0.594	5.1	0.727
	6	2.8	0.929	3.6	0.508
	7	2.4	0.815	2.5	0.352
	8	3.6	1.189	5.1	0.733
	9	2.0	0.655	4.2	0.597
Average		2.2	0.724	3.8	0.543

Cellulose Disc		DG2		DG3	
X=		-NH ₂	PAMAM	-NH ₂	PAMAM
measures of different discs	1	6.9	0.462	3.4	0.111
	2	5.2	0.344	4.2	0.137
	3	5.3	0.352	5.1	0.166
	4	5.9	0.391	3.5	0.113
	5	4.8	0.317	4.6	0.147
	6	4.6	0.309	4.9	0.158
	7	4.6	0.304	4.2	0.137
	8	4.7	0.313	3.2	0.104
	9	6.0	0.400	4.1	0.134
Average		5.3	0.354	4.1	0.133

Cellulose Disc		DG4		DG5	
X=		-NH ₂	PAMAM	-NH ₂	PAMAM
measures of different discs	1	3.7	0.058	0.8	0.007
	2	3.0	0.047	3.2	0.025
	3	3.5	0.055	1.9	0.015
	4	3.0	0.047	0.9	0.007
	5	3.3	0.052	2.5	0.019
	6	3.9	0.061	0.7	0.005
	7	3.2	0.051	0.7	0.005
	8	3.7	0.059	2.4	0.019
	9	3.3	0.053	2.5	0.020
Average		3.4	0.053	1.6	0.013

Cellulose Disc		DG6	
X=		-NH ₂	PAMAM
measures of different discs	1	0.5	0.002
	2	0.3	0.001
	3	0.7	0.003
	4	1.1	0.004
	5	1.2	0.005
	6	0.4	0.002
	7	0.3	0.001
	8	1.3	0.005
	9	0.5	0.002
Average		0.7	0.003

Determination of the number of amine groups remaining free after BPO allergenic determinant reaction at the surface of the cellulose anchored dendrimer

Cellulose Disc		DG0BPO	DG1BPO	DG2BPO	DG3BPO
X=		-NH ₂	-NH ₂	-NH ₂	-NH ₂
measures of different discs	1	2.2	0.6	0.4	0.5
	2	1.0	1.2	0.4	0.5
	3	1.7	0.8	0.2	0.5
	4	2.3	1.2	0.8	1.7
	5	1.2	1.0	0.4	1.0
Average		1.7	0.9	0.4	0.8

Cellulose Disc		DG4BPO	DG5BPO	DG6BPO
X=		-NH ₂	-NH ₂	-NH ₂
measures of different discs	1	0.1	0.1	0.3
	2	0.4	0.6	0.3
	3	0.8	1.0	0.6
	4	0.3	0.9	0.4
	5	0.1	0.6	0.5
Average		0.3	0.6	0.4