

Methodologies to anchor dendrimeric nanoconjugates to solid phase: toward an efficient in vitro detection of allergy to β -lactam antibiotics

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Abstract

β -lactam antibiotics are the most common cause of adverse drug reactions, and in vitro immunoassays are often employed to diagnose this allergy. Cellulose solid supports displaying hapten-carrier conjugates are traditionally used for quantifying specific IgE antibodies to determine whether a patient is allergic to the hapten. Our approach makes use of polyamidoamine dendrimers as carriers, and differs from traditional solid-phase activation methods by using two different haloalkanoyl halides as reagents instead of cyanogen bromide. Evaluation of the functionalization in the three different surfaces with benzylpenicilloyl-dendrimer conjugates showed a higher density of conjugates when using haloalkanoyl halides. The resulting surfaces were evaluated in vitro by radioimmunoassay using sera from patients allergic to penicillins. Selective and specific IgE recognition was observed in the different activated disks, though those activated with haloalkanoyl halides presented better sensitivity.

From the Clinical Editor: A novel and sensitive method of dendrimeric nanoconjugate-based in vitro detection of allergy to β -lactam antibiotics is presented in this basic science paper.

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Allergic reactions to β -lactams represent the primary cause of drug reactions mediated by specific immunological mechanisms. To identify penicillin IgE-mediated allergy, the radioallergosorbent test (RAST) has been employed as a complementary diagnostic method to in vivo tests, skin testing, and drug provocation tests. In this context, RAST is a test tube that measures IgE antibodies directed against a hapten (drug), which may indicate whether the patient is allergic to that drug. This technique employs solid supports bonded to hapten-carrier conjugates as materials to incubate with patient sera. To obtain

a typical support for RAST to benzylpenicillin, benzylpenicilloyl (BPO) groups are conjugated to carrier molecules, which are coupled to cyanogen bromide-activated cellulose surfaces. To obtain dense and reproducible hapten-carrier conjugates on surfaces, we have undertaken several studies involving the use of polyamidoamine (PAMAM) dendrimers based on their potential as multivalent proteinlike materials for biotechnological applications.^{1,2}

RAST inhibition studies have previously confirmed the usefulness of BPO-PAMAM as fluid-phase conjugates for the recognition of IgE from patients allergic to penicillin.³ In addition, these BPO-PAMAM conjugates attached to cellulose supports allowed clinical RAST evaluation, with validated results.⁴ Solid-phase activation was performed by using cyanogen bromide, one of the most common activation methods for solid supports in biochemistry⁵ and classically used for RAST material applications.⁶

Here we evaluate a different method to activate the cellulose surface and its influence on specific IgE molecular recognition to

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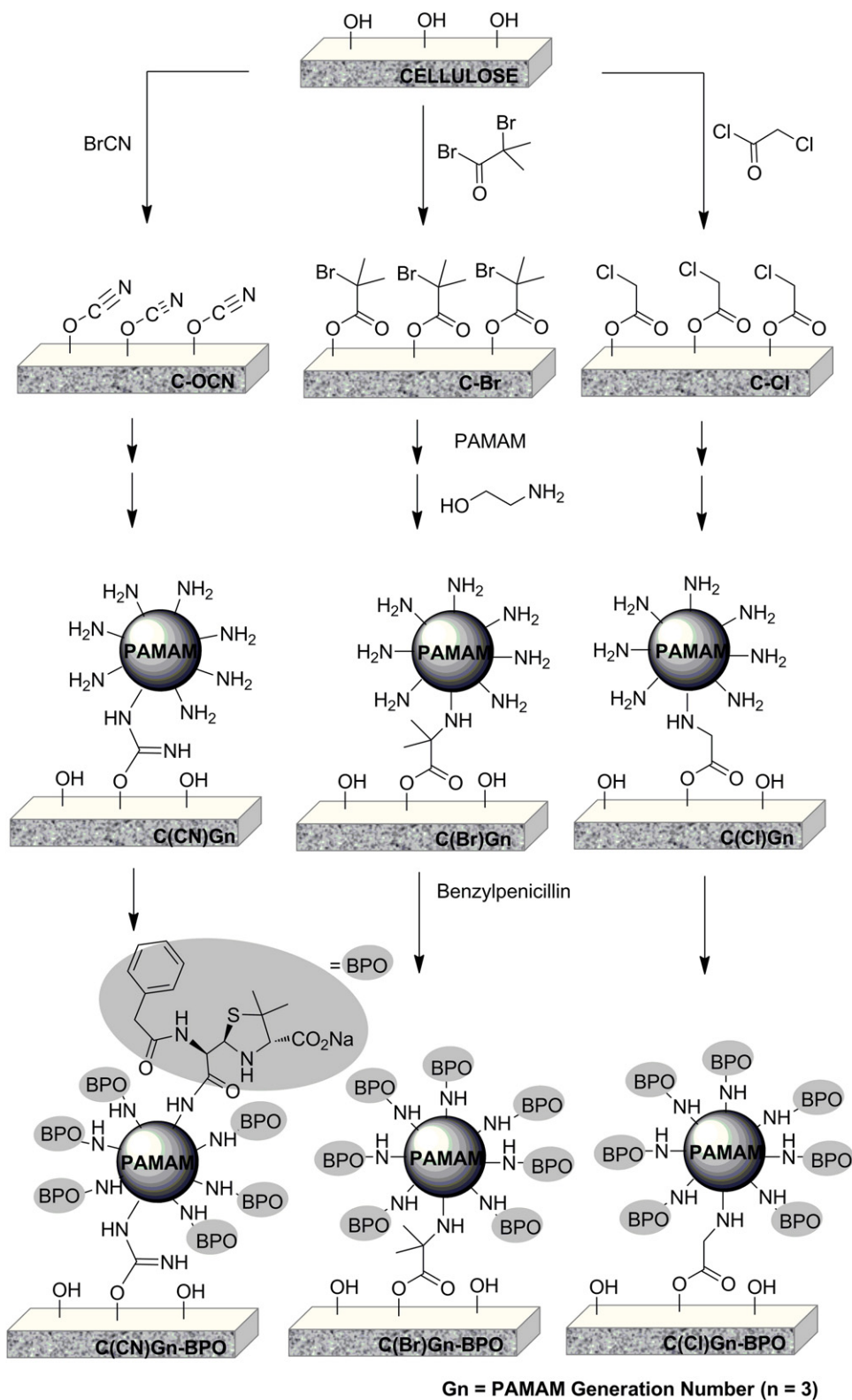


Figure 1. Cellulose surface modification methodologies to attach hapten-carrier conjugates.

β -lactams. We compared the traditional cyanogen bromide methodology with the use of haloalkanoic acid reagents to bind hapten-carrier conjugates to a cellulose solid phase. The different

functionalized supports were assessed by RAST to study their influence on the specific recognition of IgE from patients with an IgE-mediated allergic reaction to β -lactams.

Methods

Activation of solid phases to yield **(C-OCN)**⁴ and **(C-Br** and **C-Cl)**,⁷ and the following step reactions, are described in Supplementary Material, available online at <http://www.nanomedjournal.com>. The ninhydrin test for a series of disks was carried out as described elsewhere (see Supplementary Material).⁴

Selected patients had been diagnosed with an immediate (IgE-mediated) allergic reaction to penicillins by following a well-validated protocol (European Network of Drug Allergy).⁸ RAST to benzylpenicillin was performed as described elsewhere.⁹ More details are given in Supplementary Materials.

The study was conducted according to the Declaration of Helsinki principles and was approved by the Ethics Committee of Carlos Haya hospital. All subjects—patients and controls—included in the study were informed orally about the study and signed the corresponding informed consent.

Results and discussion

Cellulose disks were used as the solid phase for all experiments, given the importance of the easy handling of these materials for routine hospital assays. These surfaces were activated using three different reagents and then covalently bound to hapten-carrier conjugates, following the schemes depicted in Figure 1. A third-generation PAMAM dendrimer, with 32 surface amino groups, was chosen as the carrier to compare the different procedures, because the third- and fourth-generation dendrimers exhibit optimum RAST levels in our previous studies.⁴ The traditional methodology involved the conversion of hydroxyl into cyanate groups **(C-OCN)** and the subsequent reaction of dendrimer primary amino groups to obtain the isourea derivative via which the dendrimers anchored to the cellulose.⁴ Our newly developed procedure used haloalkanoyl halides (chloroacetyl chloride and α -bromoisobutyryl bromide), because they are bifunctional compounds and can therefore be used as a chemical linker. Both reagents were immobilized by reaction of the haloalkanoyl halide with the hydroxyl groups in the cellulose⁷ to yield **C-Br** and **C-Cl** disks. Thus, the reagents were attached to the cellulose through an ester bond, whereas the other end of the reagent molecule was able to form an amine linkage with the PAMAM dendrimers. The unreacted activated groups were neutralized by reaction with 2-aminoethanol⁴ to obtain **C(X)Gn** disks. Finally, these dendrimerized surfaces enabled the covalent immobilization of benzylpenicillin, providing BPO-PAMAM conjugates on cellulose disks.

To ensure reliability of our immunoassay, the quality of the materials was evaluated. The ninhydrin method enabled the quantitation of surface primary amine groups on the disks. Tests were conducted on a series of dendrimerized disks before and after reaction with benzylpenicillin. The results (Supplementary Figure S1) showed very homogeneous values, and therefore almost identical degrees of functionalization, for all disks within each type. These results revealed the presence of amine groups, confirming the attachment of dendrimers to the disks, and reflected reproducibility of the dendrimer coupling and functionalization methods. The averages of these

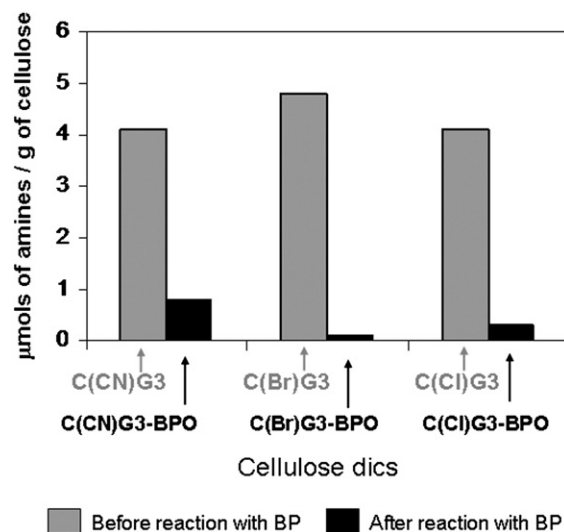


Figure 2. Results obtained from the determination of primary amino groups on the different disks.

data are shown in Figure 2. The three activation methods are useful for introducing amine groups to the disks **C(X)Gn**, although α -bromoisobutyryl bromide outperformed the other two reagents, with 4.8 μmol of amine group per gram of cellulose compared to 4.1 μmol . The few free amine groups remaining in the disk after reaction with benzylpenicillin revealed the efficiency of the dendrimer haptenization step. All these data allow estimation of the amount of both the dendrimers and the haptens attached to the cellulose disks (Supplementary Table S1). The quantity of dendrimers bound to **C(Br)G3** (0.155 $\mu\text{mol/g}$) is slightly higher than that bound to both **C(CN)G3** and **C(Cl)G3** (0.133 $\mu\text{mol/g}$). Moreover, the data indicate that the number of attached BPO groups reached a maximum value for **C(Br)G3-BPO**, followed by **C(Cl)G3-BPO** and **C(CN)G3-BPO** (Supplementary Table S1).

The resulting surfaces were validated for specific IgE recognition by RAST using sera from β -lactam-allergic patients and controls who tolerated this drug (Figure 3). As can be seen in Figure 3, A, the different disks produced positive RAST results in most of the sera from patients allergic to benzylpenicillin. Data showed a higher percentage of positivity (77.8%) with **C(Br)G3-BPO** disks, followed by **C(Cl)G3-BPO** and **C(CN)G3-BPO** (66.7% and 44.4% positivity, respectively). Significantly, **C(Br)G3-BPO** disks enabled cases 1, 5, and 6 to be correctly diagnosed as allergic to BPO. The percentage of RAST obtained with all controls gave negative values (Figure 3, B) and showed no differences among the three different disks. Figure 4 shows important differences ($P < 0.05$) for the specific IgE levels in the three types of disks when comparing the results from both groups of sera (benzylpenicillin-allergic and controls). In the allergic group, increases were observed with both **C(Br)G3-BPO** and **C(Cl)G3-BPO**, compared to **C(CN)G3-BPO**. However, no differences were seen between the results obtained with **C(Br)G3-BPO** and **C(Cl)G3-BPO** disks. Notably, the specificity reached with these disks was very high (100%), as deduced from the results obtained with the controls.

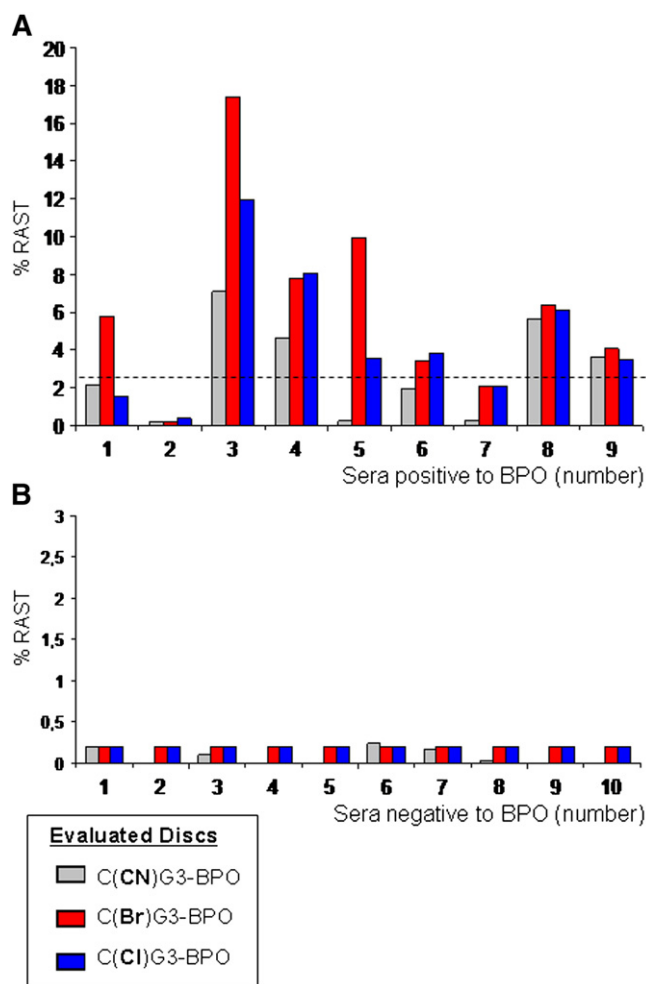


Figure 3. RAST values obtained with the three different BPO-dendrimerized disks by evaluation of 19 sera previously assessed by ImmunoCAP system (see Supplementary Material) from (A) BPO-allergic patients and (B) tolerant controls.

In conclusion, haloalkanoyl halides proved to be more efficient linkers than cyanogen bromide for attaching hapten-dendrimer conjugates onto cellulose surfaces. The resulting ester linkage should be more stable and robust than the isocyanurate bond, and the corresponding α -haloester more reactive and stable in the reaction with the amine groups of the dendrimers, being also optimized as a result of the better reactivity of the tertiary halide reagent. Thus, the methodology described provides reproducible materials and increases the density of conjugates on the surfaces obtained with the traditional methodology. Specific IgE recognition was observed in the different activated disks, presenting a higher percentage of positivity when α -bromoisobutyryl bromide was employed, followed by chloroacetyl chloride and cyanogen bromide. These in vitro sensitivity improvements are in agreement with the higher density of nanoconjugates on the surfaces. We believe this methodology may offer a significant advance in the sensitivity of in vitro radioimmunoassay-based diagnosis of drug allergy.

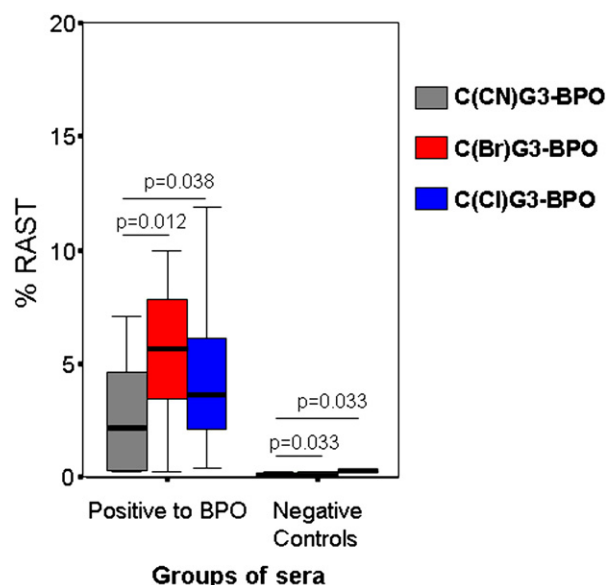


Figure 4. Comparison of percent RAST results between two groups of sera (allergic patients and tolerant controls) and the three disks.

Appendix A. Supplementary data

Supplementary data to this article can be found online at [doi:10.1016/j.nano.2011.07.008](https://doi.org/10.1016/j.nano.2011.07.008).

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