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## **Nanoarchitectures for Efficient IgE Cross-Linking on Effector Cells to Study Amoxicillin Allergy**

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**Short title: Amoxicillin-dendrimeric structures for effector cell activation**

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#### **Author Contributions**

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#### **Conflict of Interest**

The authors declare that they have no known competing financial interests or personal relationships that could appear to influence the work reported in this paper.

## ABSTRACT

**Background:** Amoxicillin (AX) is nowadays the  $\beta$ -lactam that more frequently induces immediate allergic reactions. Nevertheless, diagnosis of AX allergy is occasionally challenging due to risky *in vivo* tests and non-optimal sensitivity of *in vitro* tests. AX requires protein haptation to form multivalent conjugates with increased size to be immunogenic. Knowing adduct structural features for promoting effector cells activation would help to improve *in vitro* tests. We aimed to identify the optimal structural requirement in specific cellular degranulation to AX using well-precised nanoarchitectures of different lengths.

**Method:** We constructed eight Bidendron Antigens (BiAns) based on polyethylene glycol (PEG) linkers of different lengths (600-12000 Da), end-coupled with PolyAmidoAmine dendrons that were terminally multi-functionalized with amoxicilloyl (AXO). *In vitro* IgE recognition was studied by competitive RadioAllergo Sorbent Test (RAST) and antibody-nanoarchitecture complexes by transmission electron microscopy (TEM). Their allergenic activity was evaluated using bone marrow-derived mast cells (MCs) passively sensitized with mouse monoclonal-IgE against AX, and humanized RBL-2H3 cells sensitized with polyclonal antibodies from sera of AX-allergic patients.

**Results:** All BiAns were recognized by AX-sIgE. Dose-dependent activation responses were observed in both cellular assays, only with longer structures, containing spacers in the range of PEG 6000-12000 Da. Consistently, greater proportion of immunocomplexes and number of antibodies per complex for longer BiAns were visualized by TEM.

**Conclusions:** BiAns are valuable platforms to study the mechanism of effector cells activation. These nanomolecular tools have demonstrated the importance of the adduct size to promote effector cells activation in AX allergy, which will impact for improving *in vitro* diagnostics.

**Keywords:** Amoxicillin; Drug Allergy; IgE Cross-linking; Immunocomplex; Nanostructure.

**Abbreviations:** AX, amoxicillin; AXO, amoxicilloyl; AXO-Bu, amoxicilloyl-butylamine; BiAn, bidendron antigens; DNP, dinitrophenyl; HSA, human serum albumin; Hum, human; MCs, mast cells; MoAbs, monoclonal antibodies; MW, molecular weight; PAMAM, PolyAmidoAmine; PEG, polyethylene glycol; PLL, poly-L-lysine; RAST, radio allerge sorbent test; RBL, rat basophilic leukemia cell; SAR, structure-activity relationship; TEM, transmission electron microscopy.

## INTRODUCTION

Drug allergy accounts for 5-10% of all adverse drug reactions and could result in life-threatening complications.<sup>1,2</sup>  $\beta$ -lactam antibiotics are the most frequent triggers of reactions, with amoxicillin (AX) as the most common elicitor nowadays.<sup>3</sup>  $\beta$ -lactam allergy can be induced by different immune mechanisms, among which the IgE antibodies-mediated one is the most common and better studied.<sup>4,5</sup> The diagnosis of immediate reactions to AX is based mainly on *in vivo* and *in vitro* methods,<sup>6</sup> being drug provocation test the gold standard; although it is risky and not recommended in patients with severe reactions.<sup>2,7,8</sup> *In vitro* tests are based on the determination of specific IgE (sIgE), with the commercial ImmunoCAP only detecting 20% of allergic patients, and on the quantification of basophil activation after stimulation with the culprit drug,<sup>9,10</sup> showing sensitivity around 50%. Among the factors affecting such low sensitivity could be the fact that correct antigenic determinant and/or conjugates are not incorporated to *in vitro* tests.<sup>6,11,12</sup>

According to the hapten hypothesis, AX is a low molecular weight (MW) compound that must form protein covalent conjugates with increased size and multivalence to be immunogenic.<sup>13</sup> Spontaneous conjugation of AX occurs due to the structural propensity of its  $\beta$ -lactam ring opening by the nucleophilic primary amines from proteins that results in the amoxicilloyl (AXO) antigenic determinant.<sup>14</sup> The immunological recognition of such multivalently presented antigenic determinants on a conjugate by, at least, two adjacent IgE antibodies that are bound to their high-affinity receptor (Fc $\epsilon$ RI) on the surface of tissue mast cells (MCs) or circulating basophils, results in an intricate process of IgE cross-linking,<sup>15–18</sup> releasing preformed inflammatory mediators and eliciting the acute allergic response.<sup>15,19</sup> The efficiency of the stimulation on cell degranulation is dependent on many factors, including the drug antigenic determinant structure,<sup>18,20</sup> its valency on the conjugate or complete antigen,<sup>21,22</sup> the size of the conjugate,<sup>15,23</sup> the proximity of the IgE epitopes,<sup>24</sup> and the steric hindrance.<sup>16,24–26</sup>

The study of these complex cellular and structural restrictions in the *in vitro* activation of effector cells requires sophisticated structures that are well-defined and characterized to facilitate the interpretation of results. In this regard, different molecules have been designed to assess the influence of these parameters on the degranulation of MCs using synthetic haptens linked on bi- or multivalent structures. The most widely used experimental model utilizes the rat basophilic leukemia cell (RBL)/dinitrophenyl (DNP) and/or dansyl system, in which the RBL cells are

primed with monoclonal IgE-DNP and/or IgE-dansyl antibodies, followed by stimulation with a multivalent haptenated structure to induce degranulation.<sup>15,16,18,23,26–30</sup> Dissimilar optimal sizes have been reported for effective cross-linking as a consequence of the diverse features of the synthetic allergen systems studied, differing in the density of haptens (or valency),<sup>23,30</sup> three-dimensional structure, flexibility<sup>23</sup> or rigidity<sup>15,29,30</sup> of the structures.

However, owing to the complexity of real drug allergy scenario, this kind of Structure-Activity Relationship (SAR) approach with nanostructures has never been addressed involving drugs (as haptens) or samples from allergic patients (drug-sIgE). Inspired by our previous design of Dendrimeric Antigens that permit controlling the size, multivalence (number of haptens), and three-dimensional structure,<sup>31,32</sup> and showed potential use for drug-sIgE quantification,<sup>33–37</sup> we propose a new related nanoarchitecture design to expand the interaction studies with the immune system.

Herein, we construct a set of symmetrical dendrimer-derived nanoarchitectures, called Bidendron Antigens (BiAns), in which two dendrons decorated with multiple units of AXO are separated by flexible polymeric spacers of different lengths (Figure 1), to evaluate the influence of the nanoarchitecture size for promoting the activation of effector cells that causes drug allergic reactions. This SAR study will help understand deeply the required distance between AXO determinantsto activate basophils or MCs, which would be useful for improving the sensitivity of *in vitro* tests for diagnosing allergic reactions to AX.

## METHODS

### Patients

Patients with a clinical history of immediate allergy to AX and tolerant subjects were evaluated following the European Academy of Allergy and Clinical Immunology (EAACI) guidelines.<sup>38,39</sup> The study was conducted according to the Declaration of Helsinki principles and was approved by the Provincial Ethics Committee of Malaga. All subjects included in the study were informed orally and signed the corresponding informed consent.

For evaluation of AX-sIgE recognition, we used a pool of 14 sera from patients with positive skin tests to AX and high levels (>7%) of AX-sIgE measured by direct RadioAllergoSorbent Test (RAST). Patients data are displayed in Table S1.

Sera with high levels of total IgE, measured by ImmunoCAP ( $\geq 250$  kU/L) were selected for humanized RBL-2H3 (HumRBL-2H3) cell evaluation studies: 3 patients allergic to AX, with high levels (>7%) of AX-sIgE measured by RAST (data shown in Table S2), and 3 tolerant subjects, with no reactivity to  $\beta$ -lactams.

### Production of Conjugates and Anti-AXO Monoclonal Antibodies (MoAbs)

The synthesis, purification and characterization of the conjugate of human serum albumin (HSA) with AX (HSA-AXO) and the series of BiAn and intermediates, and the production of two hybridomae:<sup>40</sup> AO3.2, IgG<sub>2a</sub> isotype, and AO6.2, IgE isotype, are detailed in the Methods section of this article's Online Repository.

## Radioimmunoassays

Radioimmunoassays were performed by RAST as previously described<sup>14</sup> using the Poly-L-lysine (PLL) cellulose solid-phases conjugated to AX (AXO-PLL cellulose discs) and <sup>125</sup>I-anti-IgE.<sup>41</sup> Results were considered positive if they were higher than 2.5% of label uptake, which was the mean  $\pm$  2 SD of the negative control group.

Competitive inhibition immunoassays were carried out with a pool of sera with high RAST values (>7%), which was incubated with the inhibitors (AXO-butylamine (AXO-Bu) and BiAns) at four concentrations (30, 15, 3, and 0.3 mM of AXO equivalents) as the fluid phase for 18h at room temperature. Then AXO-PLL solid-phase discs were added, followed by the described RAST protocol. Results were calculated as the percentage of inhibition using the non-inhibited serum as a control.

## Transmission Electron Microscopy(TEM)

The samples were prepared according to Optimized Negative-staining (OpNS).<sup>42</sup> From the different tested MoAbs to BiAn ratios, a molar ratio of 8:1 was found to be the optimal condition. This ratio was used to incubate MoAbs with **BiAn(600)**, **BiAn(6000)**, and **BiAn(10000)** to evaluate differences in the obtained complexes as a function of the employed nanoarchitecture.

## Cell Viability and Activation Assays

Viability was assessed using [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium(MTS) assay. Bone marrow cells were collected from femurs of Balb/c mice (4-6 weeks of age) from Charles River Laboratories (Saint Germain sur l'Arbresle, Rhône, France) and cultured in DMEM with glucose and L-glutamine, fetal bovine serum, penicillin/streptomycin, and sodium pyruvate (all from Gibco, Life Technologies), plus recombinant murine SCF, IL-3, IL-9, and TGF- $\beta$  (all cytokines and growth factors were from Peprotech, Rocky Hill, NJ) to differentiate into MCs as previously described.<sup>43</sup> MCs were cultured for a minimum of 4 weeks and up to 8 weeks before they were used for functional assays. For activation through cross-linking of the IgE receptor, MCs were initially sensitized for 4h with 1  $\mu$ g/mL of mouse anti-AX IgE MoAb. After washing, MCs were resuspended in Tyrode's buffer and activated with the BiAns at 10, 50, and 100  $\mu$ M (concentration of AXO units) for 1h. A conjugate of HSA with AX at 50  $\mu$ g/mL (0.7  $\mu$ M of HSA-AXO, corresponding to 9.7  $\mu$ M of AXO)

was used as a positive control. Control experiments were performed in unsensitized cells. Experiments were performed in triplicate. All protocols involving animals followed the European legislation (Directive 2010/63/EU) and were approved by Comunidad de Madrid (Ref PROEX 089/15).

HumRBL-2H3 cells were obtained from the Cell Culture Unit of the University of Granada (Granada, Spain) and cultured in RPMI containing 10% heat-inactivated FBS, 2mM L-Glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin, in a humidified 5% CO<sub>2</sub> atmosphere at 37°C. HumRBL-2H3 cells were seeded in 96 well plates at a density of 2x10<sup>5</sup> cells/well. Confluent growing HumRBL-2H3 cells were then sensitized with serum (50% v/v) from AX allergic patients (n=3) and tolerant subjects (n=3) for 48h at 37°C. Unsensitized cells were used as controls. Subsequently, cells were stimulated with the series of BiAns at different concentrations of AXO units (1 µM, 10 µM, 20 µM, 50 µM, 100 µM, 150 µM, 200 µM) for 2h. In parallel experiments, cells were stimulated with the corresponding PEG linkers (structures without dendron-AXO), as negative controls. The HSA-AXO conjugate (at 10 µM concentration of AXO) was used as a positive control of degranulation induction.

### Quantification of $\beta$ -hexosaminidase Release

The cell degranulation response was quantified measuring the level of  $\beta$ -hexosaminidase released in culture supernatants.<sup>44</sup> The percentage of  $\beta$ -hexosaminidase release was calculated as a percentage of the total  $\beta$ -hexosaminidase content.

For further details, see the Methods section of this article's Online Repository.

## RESULTS

### Design of Dendrimer-based Nanoarchitectures

A novel set of symmetrical dendrimer-derived nanoarchitectures, BiAns, was designed, synthesized (Figure 1), purified, and characterized (Figure S1). The BiAns were constructed to display critical design features: (i) bearing multiple units of AX antigenic determinant on the periphery of the dendron scaffolds to support multivalent interaction with the target antibody; (ii) a flexible hydrophilic PEG spacer that provides a specific length between dendrons, promotes aqueous solubility,<sup>45</sup> and reduces non-specific protein adsorption, immunogenicity, and antigenicity.<sup>23,46</sup> A series of PEG chains of 8 different average MWs (range 600-12000 Da) were chosen as linkers. Commercially available second-generation (G<sub>2</sub>) PolyAmidoAmine (PAMAM) dendron was selected as a common multivalent scaffold as it displays peripheral amino groups that covalently bind to AX in an efficient manner, and previous studies showed that Dendritic Antigens based on PAMAM are recognized by penicillin-sIgE.<sup>32,33,35,36</sup> The resulting BiAns encompass the same number of peripheral functionality (16 AXO per BiAn), differing only in the length of the hydrophilic spacer separating the dendrons, ranging in aqueous solution from 3 to 20 nm, with a maximal possible extension from 4.8 to 95.5 nm (Table 1).

### IgE Recognition of BiAns

The ability of AX-sIgE to recognize BiAn conjugates was evaluated by competitive RAST inhibition immunoassays using sera from AX-allergic patients. This assay consists in competitive serum IgE recognition between the solid phase (AXO-PLL conjugate attached to cellulose discs) and the different inhibitors, containing AXO at different concentrations, in the fluid phase, and results are represented as % inhibition IgE binding (Figure 2). A butylamine-AXO monomeric conjugate was also employed as a control inhibitor.<sup>14</sup> Assays were performed at equimolar amounts of AXO for all the conjugates, with a maximum concentration not higher than 30 mM of AXO, due to solubility issues at higher concentrations of BiAns. At such maximum concentration, sera were inhibited by all BiAns. In general, inhibition dropped at 3 and 0.3 mM of AXO in inhibitors. The presented data supports that all compounds are recognized by AX-sIgE in a concentration-dependent manner, with no differences on the degree of IgE recognition among the different BiAns, as they present the same number of AXO equivalents (Figure 2).

## Antibody-nanoarchitectureComplexes

To visualize IgE binding to the BiAn nanoarchitectures and the shape of the resulting immunocomplex, OpNS TEM was performed.<sup>47,48</sup> Optimization of the staining and imaging conditions with MoAb alone, AO3.2 (specific to AXO structure), showed Y-shaped monomeric antibodies in different orientations, (Figure 3A) and their corresponding two  $F_{ab}$  and one  $F_c$  domains (Figure 3B).

To study the influence of spacer length of the BiAns on the formation of immunocomplexes, various nanoarchitectures (**BiAn(600)**, **BiAn(6000)**, and **BiAn(10000)**) were incubated with the antibody prior to evaluation of the complexes by OpNS TEM. In all cases, the immunocomplex formation followed a similar pattern, with most of the observed particles corresponding to individual antibodies (60-83%). Since the attached nanoarchitectures were not detectable, it was not possible to distinguish monomeric complexes from non-complexed antibodies. Considering only structures with more than one antibody per particle, the most predominant configuration corresponded to dimeric complexes, which appeared to be the most energetically favored structure (Figure 3C, Table S3). Since the length of the PEG spacer in the BiAn increased (PEG 10000 > 6000 > 600), the percentage of complexes with 2 or more than 2 antibodies also increased. This might be ascribed to a larger steric hindrance in the structures with shorter PEG spacer, where the two AXO-dendrons are much closer to each other, preventing the binding of other antibodies to form the immunocomplex.

Besides the number of antibodies per complex, other differences could be also detected regarding the morphology of the complexes as a function of the studied molecule (Figure 3D). For complexes involving 4 antibodies with an open structure, up to 3 antibodies could be found to bind on a common spot (which could contain both dendrons of one BiAn). For larger complexes, conformational elucidation becomes extremely challenging due to the superposition of several antibodies. It is important to bear in mind that the antibody saturation conditions of the experiment may favor the formation of ring-closed complexes, and therefore minimize cross-linking potential. In this context, it is worth noting that all the complexes observed with **BiAn(600)** presented an open or linear structure (Figure 3D), which in the case of 2:2 complexes might correlate with the too-short spacer between both antigenic dendrons that impedes the simultaneous interaction with both recognition sites of a single antibody (Table 1), estimated in 11-13 nm.<sup>49</sup> On the other hand, and although open structures were still predominant, Figure 3D also shows some examples of ring-

closed complexes with different numbers of antibodies per complex that were observed with **BiAn(6000)**(6.3 % of the complexes) and **BiAn(10000)** (6.2 %).

### Toxicity Assay

Effect of different concentrations of BiAns on HumRBL-2H3 cell viability revealed a survival rate greater than 70% at most concentrations assayed. Only the highest one (200  $\mu$ M) in **BiAn(2000)** and **BiAn(4000)** reduced the HumRBL-2H3 viability up to 64% (data not shown).

### Effects of BiAns on IgE Activation of Bone Marrow-derived Cells

The capacity of nanoarchitectures to induce IgE-dependent degranulation of bone marrow-derived MCs was evaluated. For this, cells were sensitized with IgE MoAb against AX (specific to AX side chain), then treated with different concentrations of BiAns (in terms of the same equivalents of AXO), and afterwards,  $\beta$ -hexosaminidase assay was performed (Figure 4, left). Stimulation of cells with HSA-AXO (10  $\mu$ M of AXO moieties) induced up to 25% of  $\beta$ -hexosaminidase release. However, cell stimulation with BiAns using an equivalent concentration of AXO only caused high activation with the **BiAn(10000)**, inducing 17% of  $\beta$ -hexosaminidase release. Only BiAns constructed with PEG of MW  $\geq$  6000 Da induced cell degranulation, and in a dose-dependent manner, showing up to 19% of  $\beta$ -hexosaminidase released at higher concentrations (50  $\mu$ M and 100  $\mu$ M of AXO moieties), and bringing out the importance of the polymeric spacer length to achieve cell activation (Figure 4B). None of the BiAns tested induced cell degranulation on unsensitized cells (Figure 4A), indicating that activation occurs through an IgE mechanism.

### Effect of BiAns on IgE-Induced Degranulation in RBL-2H3 Cells.

Next, we chose the HumRBL-2H3 cell line for evaluation, which shares some characteristics with both MCs and basophils, and expresses human IgE receptor (Fc $\epsilon$ RI).<sup>27</sup> Cells were primed with polyclonal antibodies from sera of AX-allergic patients and tolerant subjects, then sensitized RBL-2H3 cells were treated with different concentrations of BiAns (in terms of the same equivalents of AXO determinants), and subsequently,  $\beta$ -hexosaminidase assay was performed. Results, shown in Figure 4 right, indicate that sensitized cells with sera from allergic patients and stimulated with BiAns containing PEG of MW  $\geq$  6000 Da significantly induced the  $\beta$ -

hexosaminidase release in a concentration-dependent manner, compared with negative control groups (PBS and HSA activated cells)(Figure 4B bottom, Figure S3). Blank structure controls (PEGs) did not induce cell degranulation (Figure S3). Moreover, the highest concentrations of BiAns containing  $MW \geq 6000$  Da induced a release of 33% of  $\beta$ -hexosaminidase, similar to the one induced by HSA-AXO (at 10  $\mu$ M conc of AXO)(Figure 4B). Among these, **BiAn(10000)** and **BiAn(12000)** are the most effective intermolecular cross-linker as they induced above 27% of  $\beta$ -hexosaminidase release at 10, 50, and 100  $\mu$ M of AXO, although at lower concentration (1  $\mu$ M) only **BiAn(10000)** induced such substantial degranulation (Figure S3). No significant differences were observed in these BiAns treatments when the cells were sensitized with sera from tolerant subjects (Figure 4B top), and on unsensitized cells included as control of IgE activation (data not shown); whereas a more specific dose-response effect was observed when the cells were sensitized with sera from patients. The fact that none of the BiAns induced cell degranulation on unsensitized cells excludes direct activation by off-target occupancy of cell surface receptors. In addition, the absence of activation observed in parallel experiments with HMC 1.2 cell line, which exhibited a similar phenotype to that of human mast cells, expressing IgG receptor (Fc $\gamma$ R) but not the high-affinity IgE receptor (Fc $\epsilon$ R),<sup>50</sup> demonstrated that the BiAns do not trigger degranulation by an IgG-mediated pathway (Figure S4) suggesting that BiAns stimulate the degranulation on HumRBL-2H3 through an IgE pathway.

## DISCUSSION

Our SAR study with well-characterized BiAns shows that the size and the proximity of AXO-determinants on these conjugates influence the number and shape of immunocomplexes and their subsequent ability to activate *in vitro* effector cells in an IgE-dependent mechanism. Through competitive immunoassays, it could be shown that all BiAns were recognized by AX-sIgE from patients. The higher concentration of determinants (AXO) increased the extent of inhibition, a pattern already reported with the monomeric AXO-Bu conjugate<sup>14</sup> and Dendrimeric Antigens with different penicillin determinants.<sup>32</sup> The increased inhibition obtained with the monomeric AXO-Bu, compared with BiAns, can be attributed to the absence of steric interactions for sIgE binding to AXO moieties in this small conjugate. In BiAn, PEG chains could contribute to a steric hindrance to the IgE binding, and also the high proximity of the eight AXO determinants exposed in each dendron would hinder their simultaneous IgE-recognition. Using OpNS TEM, immunocomplexes could be visualized for different BiAn sizes. Assessment of BiAn immunogenicity at cellular level reveal that IgE-mediated degranulation of bone marrow derived-MCs and HumRBL-2H3 cells with BiAns is polymeric spacer length dependent. In both cellular assays, dose-dependent activation responses were observed with all BiAn containing flexible linkers above a critical size (PEG 6000). Although these PEG polymers take on a spherical equilibrium configuration in an aqueous environment (for instance 13 nm for PEG 6000 in its folded conformation), their chain units move freely<sup>51</sup> and both ends could be at any position within the contour length of the polymer chain (~48 nm) (Table 1). Similar levels of activation were found between BiAn(10000) or BiAn(12000) compared with HSA-AXO activation at the same concentration of AXO in the RBL assays. However, in the case of MC, the same level of activation is only achieved with higher concentrations of BiAns. That could be explained by the better accessibility of AXO moieties on HSA-AXO which could allow the crosslinking of more than two IgE with a single molecule. On the other hand, given the proximity among the AXO units attached to the same dendron moiety, it is unlikely that more than two IgE are bound to each BiAn during crosslinking. OpNS TEM data support cellular activation results, since the BiAn that leads to a greater proportion of immunocomplexes, and the largest number of antibodies per complex, was also the most successful one in activating cellular responses, in agreement with reported potent degranulation achieved by synthetic allergens with a valency  $\geq 3$ .<sup>29,30,52</sup>

The failure of BiAn containing PEG  $\leq 4000$  to stimulate degranulation indicates that, despite their recognition by AX-sIgE, they are inefficient at cross-linking cell surface IgE, which could be explained by a relatively low abundance of extended conformation of the PEG polymers in aqueous solution. BiAn containing the larger PEG length, and bearing equal valency, facilitates this interaction, presenting **BiAn(6000)** the minimal distance between haptens that can induce cell degranulation. The most potent stimulator **BiAn(10000)** is effective at all studied concentrations in both cell lines, MC and RBL-2H3 (as low as 1  $\mu$ M for RBL-2H3) (FigureS3), indicating that an estimated distance of  $\sim 18$  nm between haptens seems to be optimal for cross-linking the receptors. Consistent with the literature, this  $\sim 20$  nm dimension was found to be the optimal distance between DNP haptens on a rigid nanoparticle system to induce MC degranulation.<sup>30</sup> However, this is not in agreement with the pattern observed in DNP divalent and trivalent systems, in which rigid spacers of 4–5 nm stimulate stronger degranulation responses compared with those possessing spacing greater than 7–10 nm.<sup>15,29</sup> Our findings indicate that not only the size and multivalence of nanostructures are important factors for inducing degranulation, but also their flexibility.

Overall, these results suggest that BiAn nanoarchitectures containing longer PEG chains (MW range: 6000–12000 Da) are effective triggers, whereas bivalent structures of DNP, in which haptens are also separated by flexible PEG of different lengths (MW range: 400–10000 Da), were reported not to activate MC, but to behave as inhibitors.<sup>23</sup> This inhibition was explained by a preferential formation of intramolecular cross-linking of antibody by bivalent DNP of sufficient PEG length (10 nm) (stable 1:1 complexes),<sup>23</sup> or formation of cyclic dimers of IgE- Fc $\epsilon$ R1 on the cell surface with shorter linkers ( $< 5$  nm).<sup>28</sup> Comparisons in terms of chemical structure between BiAn and DNP-PEG systems<sup>23</sup> points to multivalent vs. bivalent hapten presentation as the main difference. Likely, the multivalence of the dendron in BiAn favored IgE interaction, upon dendritic or synergetic effect, and therefore the degranulation. This is in agreement with cell activation induced by other multivalent systems: dendrimers presenting 16 units of DNP induced MCs degranulation in DNP studies interaction;<sup>23</sup> different penicillin Dendrimeric Antigens activated basophils from patients, with increased stimulation index observed for those displaying higher valence (64 vs. 16 haptens);<sup>53</sup> other rigid systems, nanoparticles ( $\geq 19.8$  nm) functionalized with multiple DNP, showed to be very effective effectors, however, a reduced hapten density inhibited degranulation.<sup>30</sup>

The scenario for MC and basophil degranulation is very complex, and the use of defined nanoarchitectures has allowed the identification of the minimal requirements for their activation in a realistic model. In this regard, multivalent dendritic presentation and distance between the haptens in **BiAn** constructed with PEG MW range 6000-12000 fulfilled the optimal requirement to overcome the intricate cellular preconditions leading to cell activation. The optimal distance between AXO determinants for effective cross-linking is observed in **BiAn(10000)**.

In summary, using multivalent AXO-dendrons spaced by flexible PEG polymers, this study sheds light on the mechanism of the effector cells activation from a unique realistic perspective, using human samples and haptens in clinical use. Moreover, the synthesis of **BiAn** platform is versatile and could apply to different drug haptens or allergen epitopes. Understanding the biology and nanoscale organization of the cell membrane receptors can lead to the development of novel diagnostic and therapeutic tools for drug allergy.

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**Table 1.** Estimated and experimental values of synthetic BiAns.

| Synthetic Antigen | Estimated MW (Da) | Linker PEG MW (Da) | (C <sub>2</sub> H <sub>4</sub> O) <sub>N</sub> where N is | Flory Radius(nm) | Solution PEG length (nm)** | Extended PEG length: Contour Length(nm)*** | D (m <sup>2</sup> s <sup>-1</sup> ) | Valency |
|-------------------|-------------------|--------------------|-----------------------------------------------------------|------------------|----------------------------|--------------------------------------------|-------------------------------------|---------|
| <i>BiAn 600</i>   | 10471.9           | 600                | 14                                                        | 1.68             | 3.36                       | 4.8                                        | 1.53x10 <sup>-10</sup>              | 16      |
| <i>BiAn 1000</i>  | 10871.9           | 1000               | 23                                                        | 2.28             | 4.56                       | 8.0                                        | 1.32x10 <sup>-10</sup>              | 16      |
| <i>BiAn 2000</i>  | 11871.9           | 2000               | 45                                                        | 3.46             | 6.92                       | 15.9                                       | 1.29x10 <sup>-10</sup>              | 16      |
| <i>BiAn 4000</i>  | 13871.9           | 4000               | 91                                                        | 5.24             | 10.48                      | 31.8                                       | 1.24x10 <sup>-10</sup>              | 16      |
| <i>BiAn 6000</i>  | 15871.9           | 6000               | 136                                                       | 6.68             | 13.36                      | 47.7                                       | 1.09x10 <sup>-10</sup>              | 16      |
| <i>BiAn 8000</i>  | 17871.9           | 8000               | 182                                                       | 7.94             | 15.88                      | 63.6                                       | 9.64x10 <sup>-11</sup>              | 16      |
| <i>BiAn 10000</i> | 19871.9           | 10000              | 227                                                       | 9.08             | 18.16                      | 79.5                                       | 9.57x10 <sup>-11</sup>              | 16      |
| <i>BiAn 12000</i> | 21871.9           | 12000              | 273                                                       | 10.13            | 20.26                      | 95.5                                       | 9.50x10 <sup>-11</sup>              | 16      |

\*The Flory radius (RF) is calculated with  $RF = a \cdot N^{1/5}$  (where  $a$  is the length of a monomer unit, and  $N$  is the number of repeating monomeric units).\*\* Solution PEG length is calculated as the diameter of the polymer in aqueous solution based on Flory radius. \*\*\*The contour length (full extended length) is calculated as a product of the polymeric length ( $N$ ) and length of the monomeric unit, 3.5 Å for PEG 48.

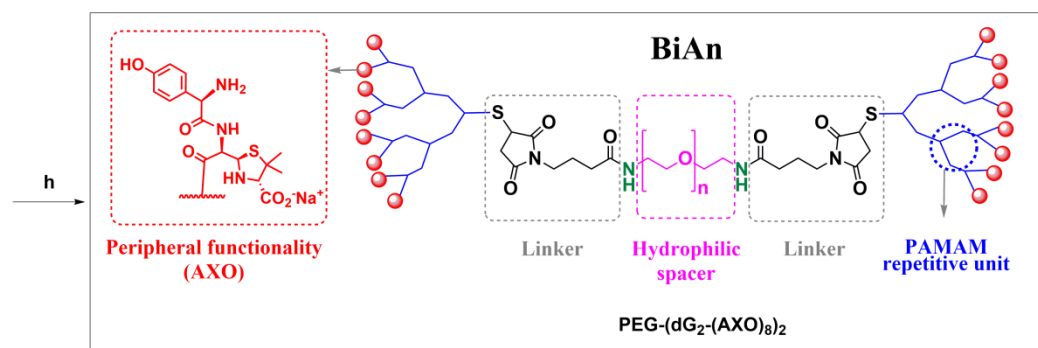
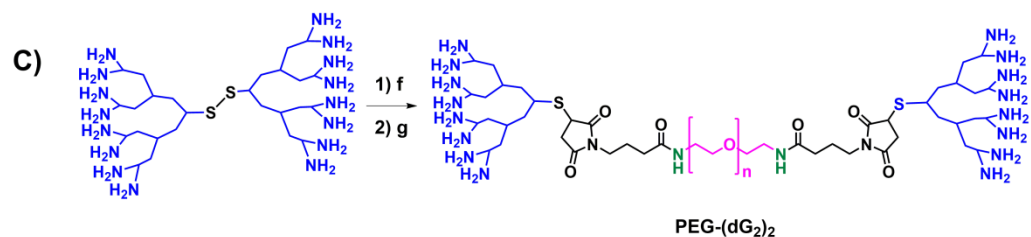
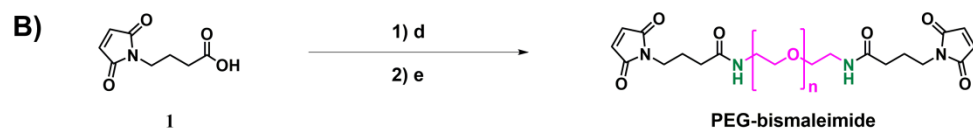
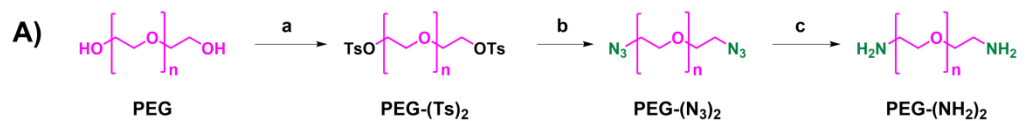
## Figure legends

**Figure 1.** General synthetic scheme including: **A)** the intermediate PEG diamine; **B)** the intermediate PEG-bismaleimide; and **C)** the final BiAns, nanoarchitectures bearing 16 AXO determinants. The structural variation among different BiAns depends on the PEG length (with  $n$  ranging from 14 to 273). Briefly, the synthetic procedure consisted in obtaining bismaleimide-activated PEG compounds that allowed coupling between thiol-core dendrons (G2), whose peripheral groups were eventually functionalized with AX. In building up this, series of independent chemical reactions were followed, and the completion of the reactions was monitored by  $^1\text{H}$  and  $^{13}\text{C}$  NMR through the appearances and disappearances of distinctive signals. Reaction conditions: (a)  $\text{TsCl}$ ,  $\text{CH}_2\text{Cl}_2$ ,  $\text{KOH}$ ,  $0\text{ }^\circ\text{C}$ ; (b)  $\text{NaN}_3$ ,  $\text{THF-H}_2\text{O}$  (80:20), reflux at  $70\text{ }^\circ\text{C}$ ; (c)  $\text{H}_2$ ,  $\text{Pd/C}$ ,  $\text{MeOH}$ , (d)  $^i\text{BuCOCl}$ ,  $\text{NMM}$ ,  $\text{CH}_2\text{Cl}_2$ ,  $0\text{ }^\circ\text{C}$ ; (e)  $\text{PEG-(NH}_2)_2$ ,  $\text{CH}_2\text{Cl}_2$ ; (f)  $\text{PBS}$ ,  $\text{pH } 6.5$ ,  $\text{TCEP}$ ; (g)  $\text{PEG-bismaleimide}$ ,  $\text{H}_2\text{O}$ ,  $\text{DMSO}$ ; (h)  $\text{AX}$ , carbonate buffer ( $0.05\text{M}$ ,  $\text{pH } 10$ ).

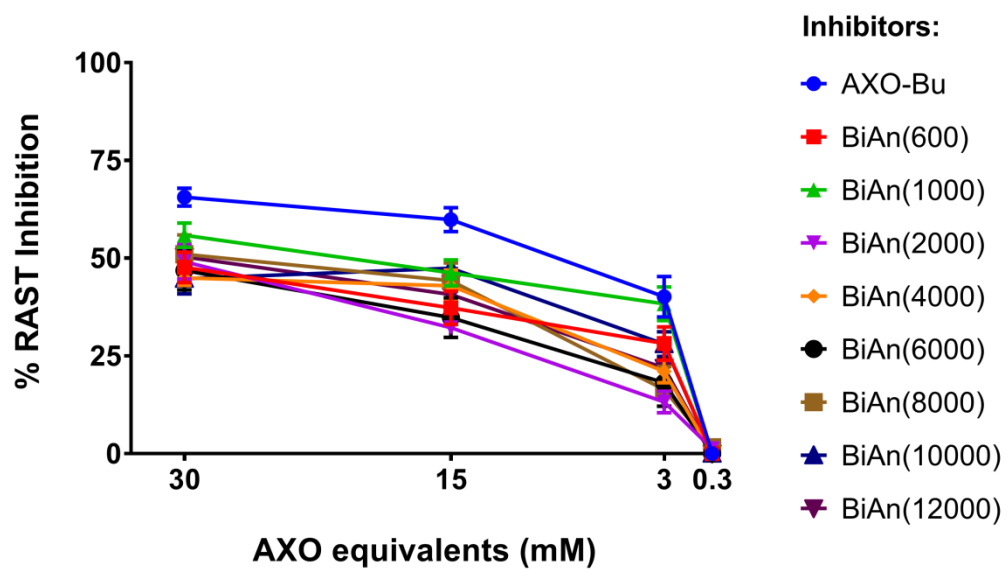
**Figure 2.** RAST inhibition assays performed with a pool of sera from patients allergic to AX, using the series of BiAns and a butylamine-AXO conjugate as inhibitors and cellulose discs modified with AXO-PLL as the solid phase. Specific IgE recognition is considered with inhibition of  $\geq 50\%$ .

**Figure 3.** Transmission electron micrograph of negatively stained MoAbs: **A)** unbound MoAbs; **B)** Zoomed-in views of selected individual antibody images. Scale bars represent  $10\text{ nm}$ ; **C)** Number of MoAbs per complex after incubation with **BiAn(600)**, **BiAn(6000)**, or **BiAn(10000)**; **D)** Zoomed-in views of selected individual complex images showing different open and ring-closed structures. Bars represent  $10\text{ nm}$ .

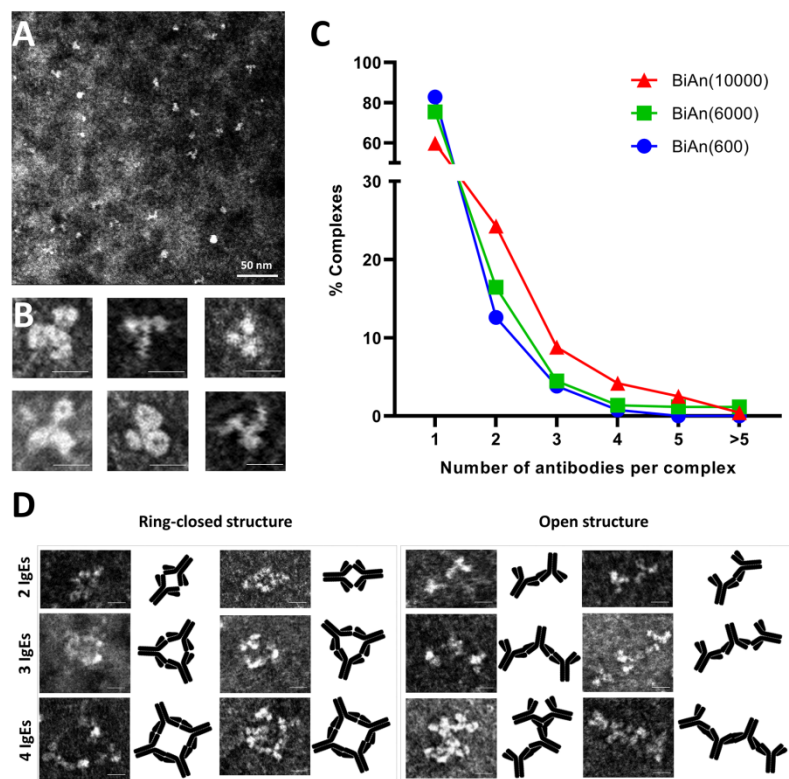
**Figure 4.** Degranulation assays after incubation of cells with the series of BiAns at  $10$ ,  $50$ , and  $100\text{ }\mu\text{M}$  of AXO equivalents. HSA and HSA-AXO (at  $10\text{ }\mu\text{M}$  of AXO) were used as negative and positive control, respectively. **A)** Percentage of  $\beta$ -hexosaminidase release in unsensitized (top) and sensitized (bottom) bone marrow-derived MCs; **B)** Percentage of  $\beta$ -hexosaminidase released by sensitized cells with sera from tolerant subjects (top) or with sera from AX allergic patients (bottom). Data are expressed as means  $\pm$  S.D. The baseline of percentage of  $\beta$ -hexosaminidase release is represented by the dotted horizontal line.



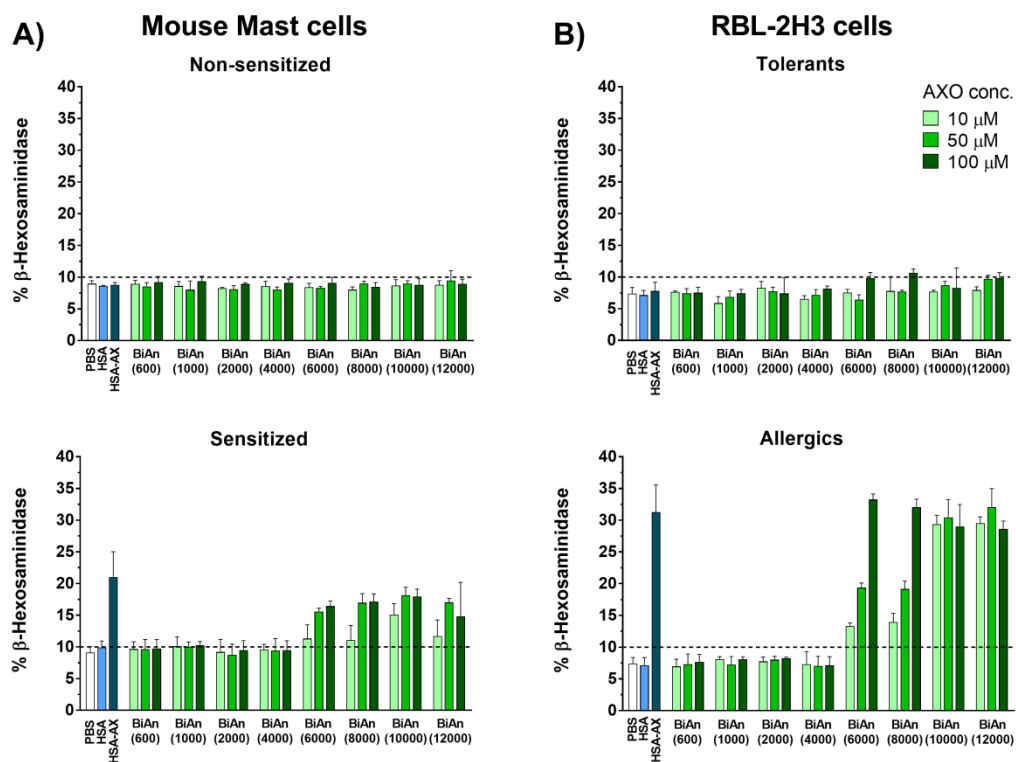
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## Online Supplementary. Tables, Figures and Methods

# Nanoarchitectures for Efficient IgE Cross-Linking on Effector Cells to Study Amoxicillin Allergy

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Torres and Montañez contributed equally to this work.

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## 1. Online Supplementary Tables and Figures

**Table S1.** Clinical characteristics of allergic patients and skin test results with penicillin antigenic determinants (BP-OL: benzylpenicilloyl-octa-L-lysine, MD: Minor Determinant, AX: Amoxicillin) and in vitro determination of sIgE by RadioAllergoSorbent Test (RAST).

| Case | Age | Sex | Clinical characteristics |                                   |                    | <i>In vivo</i> tests |           |           |     | <i>In vitro</i> tests |
|------|-----|-----|--------------------------|-----------------------------------|--------------------|----------------------|-----------|-----------|-----|-----------------------|
|      |     |     |                          |                                   |                    | Skin tests           |           |           | DPT | AX-sIgE<br>(% RAST)   |
|      |     |     | Clinical reaction        | Drugs involved<br>in the reaction | Grade<br>severity* | BP-OL                | MD        | AX        | AX  |                       |
| 1    | 51  | F   | Anaphylactic shock       | AX                                | III                | Neg                  | Neg       | Pos (IDT) | NP  | 55                    |
| 2    | 40  | M   | Anaphylaxis              | AX                                | II                 | Neg                  | Neg       | Pos (IDT) | NP  | 66                    |
| 3    | 29  | F   | Urticaria                | AX                                | I                  | Neg                  | Neg       | Pos (SPT) | NP  | 7                     |
| 4    | 35  | F   | Urticaria and Angioedema | AX-CLV                            | I                  | Neg                  | Neg       | Neg       | Pos | 12                    |
| 5    | 43  | F   | Anaphylaxis              | AX                                | II                 | Neg                  | Neg       | Pos (IDT) | NP  | 7                     |
| 6    | 59  | M   | Anaphylaxis              | AX-CLV                            | I                  | Neg                  | Neg       | Pos (SPT) | NP  | 29                    |
| 7    | 60  | F   | Anaphylaxis              | AX-CLV                            | II                 | Neg                  | Pos (IDT) | Neg       | Pos | 36                    |
| 8    | 46  | F   | Anaphylactic shock       | AX-CLV                            | III                | Neg                  | Neg       | Pos (IDT) | NP  | 73                    |
| 9    | 61  | F   | Anaphylactic shock       | AX-CLV                            | III                | Neg                  | Neg       | Pos (IDT) | NP  | 33                    |
| 10   | 61  | F   | Anaphylactic shock       | AX                                | III                | Neg                  | Neg       | Pos (IDT) | NP  | 10                    |
| 11   | 25  | M   | Anaphylaxis              | AX-CLV                            | II                 | Neg                  | Neg       | Pos (IDT) | NP  | 12                    |
| 12   | 49  | M   | Anaphylaxis              | AX-CLV                            | II                 | Neg                  | Neg       | Neg       | Pos | 33                    |
| 13   | 44  | M   | Anaphylactic shock       | AX                                | III                | Neg                  | Neg       | Pos (SPT) | NP  | 7                     |
| 14   | 40  | F   | Anaphylaxis              | AX-CLV                            | II                 | Pos (SPT)            | Neg       | Neg       | Pos | 7                     |

CLV: potassium clavulanate; DPT: drug provocation test; F: female; IDT: intradermal test; M: male; Neg: negative; NP, not performed; Pos: positive; SPT: skin prick test. \*Grading system for generalized hypersensitivity reactions: I, mild (skin and subcutaneous issues only); II, moderate (features suggesting respiratory, cardiovascular, or gastrointestinal involvement); III, severe (hypoxia, hypotension, or neurologic compromise). REF: Brown SG. Clinical features and severity grading of anaphylaxis. *J Allergy Clin Immunol* 2004;114:371-6.

**Table S2.** Clinical characteristics of allergic patients and skin test results with penicillin antigenic determinants ((BP-OL: benzylpenicilloyl-octa-L-lysine, MD: Minor Determinant, AX: Amoxicillin) and *in vitro* determination of sIgE to AX and benzylpenicillin (BP).

| Clinical characteristics |     |     |                          |                                |                 | In vivo test |     |           |     | In vitro test     |         |                  |           |                          |     |        |
|--------------------------|-----|-----|--------------------------|--------------------------------|-----------------|--------------|-----|-----------|-----|-------------------|---------|------------------|-----------|--------------------------|-----|--------|
|                          |     |     |                          |                                |                 | Skin Tests   |     |           | DPT | IgE determination |         |                  |           | Basophil activation test |     |        |
| Case                     | Age | Sex | Clinical reaction        | Drugs involved in the reaction | Grade Severity* | BP-OL        | MD  | AX        | AX  | RAST (%)          |         | ImmunoCAP (kU/L) |           | BP                       | AX  | AX/CLV |
|                          |     |     |                          |                                |                 |              |     |           |     | BP-sIgE           | AX-sIgE | AX-sIgE          | Total IgE |                          |     |        |
| 15                       | 54  | F   | Urticaria and angioedema | AX/CLV                         | I               | Neg          | Neg | Pos (IDT) | NP  | 15                | 19      | 1.95             | 392       | Pos                      | Pos | Pos    |
| 16                       | 56  | F   | Anaphylaxis              | AX/CLV                         | II              | Neg          | Neg | Pos (IDT) | NP  | 26                | 21      | 3.72             | 1158      | Neg                      | Neg | Neg    |
| 17                       | 50  | F   | Anaphylactic shock       | Unknown                        | III             | Pos (IDT)    | Neg | Neg       | Pos | 16                | 19      | 4.33             | 3802      | Neg                      | Neg | Neg    |

CLV: potassium clavulanate; DPT: drug provocation test; F: Female; IDT: intradermal test; M: male; Neg: negative; Pos: positive; NP, not performed. \*Grading system for generalized hypersensitivity reactions: I, mild (skin and subcutaneous issues only); II, moderate (features suggesting respiratory, cardiovascular, or gastrointestinal involvement); III, severe (hypoxia, hypotension, or neurologic compromise). REF: Brown SG. Clinical features and severity grading of anaphylaxis. *J Allergy Clin Immunol* 2004;114:371-6.

**Table S3.** Number (and percentage) of immunocomplexes observed with the different number of antibodies per complex as a function of the employed nanostructure.

| Samples            | Type of Immune Complexes |            |           |            |            |          |
|--------------------|--------------------------|------------|-----------|------------|------------|----------|
|                    | Monomeric                | Dimeric    | Trimeric  | Tetrameric | Pentameric | Others   |
| <b>BiAn(600)</b>   | 217 (82.8%)              | 33 (12.6%) | 10 (3.8%) | 2 (0.8%)   | -          | -        |
| <b>BiAn(6000)</b>  | 389 (75.4%)              | 85 (16.5%) | 23 (4.5%) | 7 (1.4%)   | 6 (1.2%)   | 6 (1.2%) |
| <b>BiAn(10000)</b> | 143 (59.8%)              | 58 (24.3%) | 21 (8.8%) | 10 (4.2%)  | 6 (2.5%)   | 1 (0.4%) |

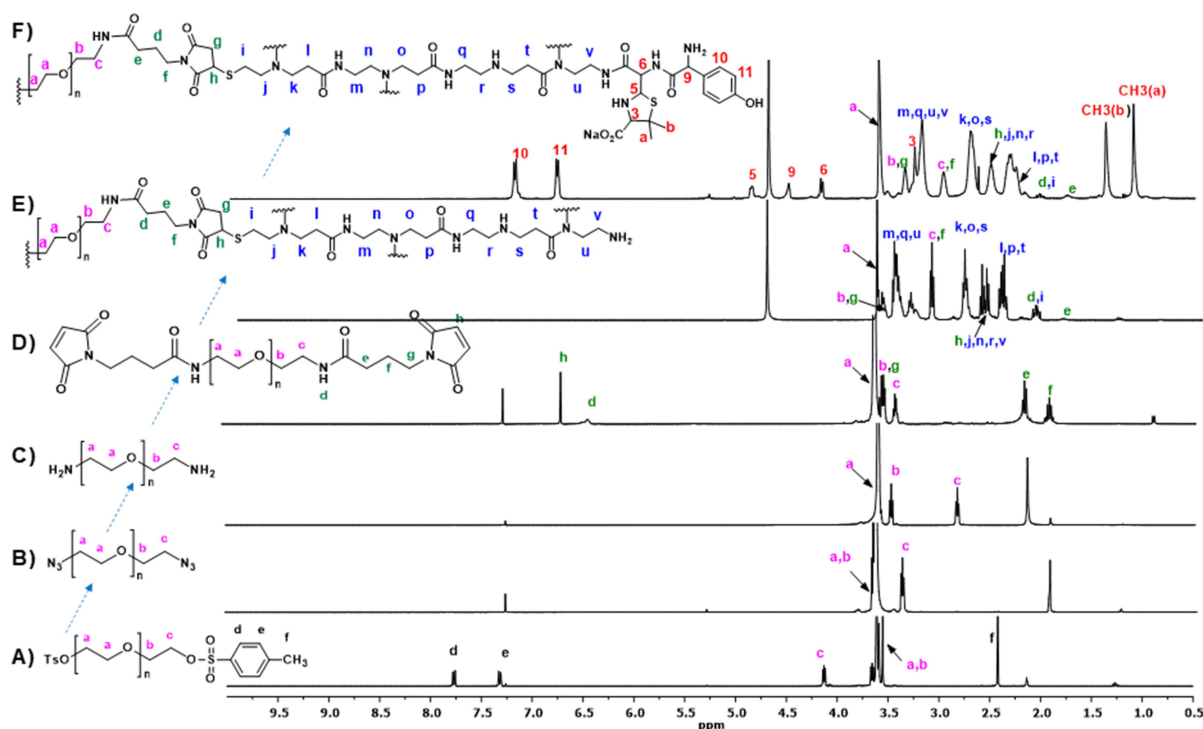


**Figure S1.**  $^1\text{H}$  NMR spectra for monitoring the reactions by following the sequential appearance and disappearance of peaks characteristic of the respective functional groups. The capital letter indicates the sequential steps in the reaction process and whereas the small letters indicate the changes in the signals assigned to the structures and corresponding to the NMR signals. **A-B**, the nucleophilic substitution of the tosylate leaving groups by azide is evidenced by the complete disappearance of peaks at around 7.78, 7.31, and 2.42 ppm, as well as the shift of the directly bonded methylene peak to the higher field (from around 4.12 to 3.37), indicating the successful synthesis of series of different MW PEG-( $\text{N}_3$ )<sub>2</sub>. **B-C**, for the synthesis of PEG diamine (PEG-( $\text{NH}_2$ )<sub>2</sub>) the  $^1\text{H}$  NMR confirms the transformation of azide to amine moieties by the shift of the two ethylene peaks in the extremes of PEG chains from around 3.65 and 3.38 ppm to 3.48 and 2.83 ppm, respectively. **C-D**, the appearance of new peaks at 6.43 and 6.72 ppm, corresponding to the presence of amide protons and the alkene of maleimide, respectively. **D-E**, disappearance of the alkene characteristic resonance at 6.72 ppm and appearance of typical signals corresponding to dendron ranging from 2.27 to 3.50 ppm. **E-F**, the resonance shift in the methylene protons of dendron linked to amine from 2.63 to 3.44 ppm indicates the formation of an amide bond and the shift and separation of  $\beta$ -lactam proton (H5 and H6) peaks indicate the opening of this AX ring.

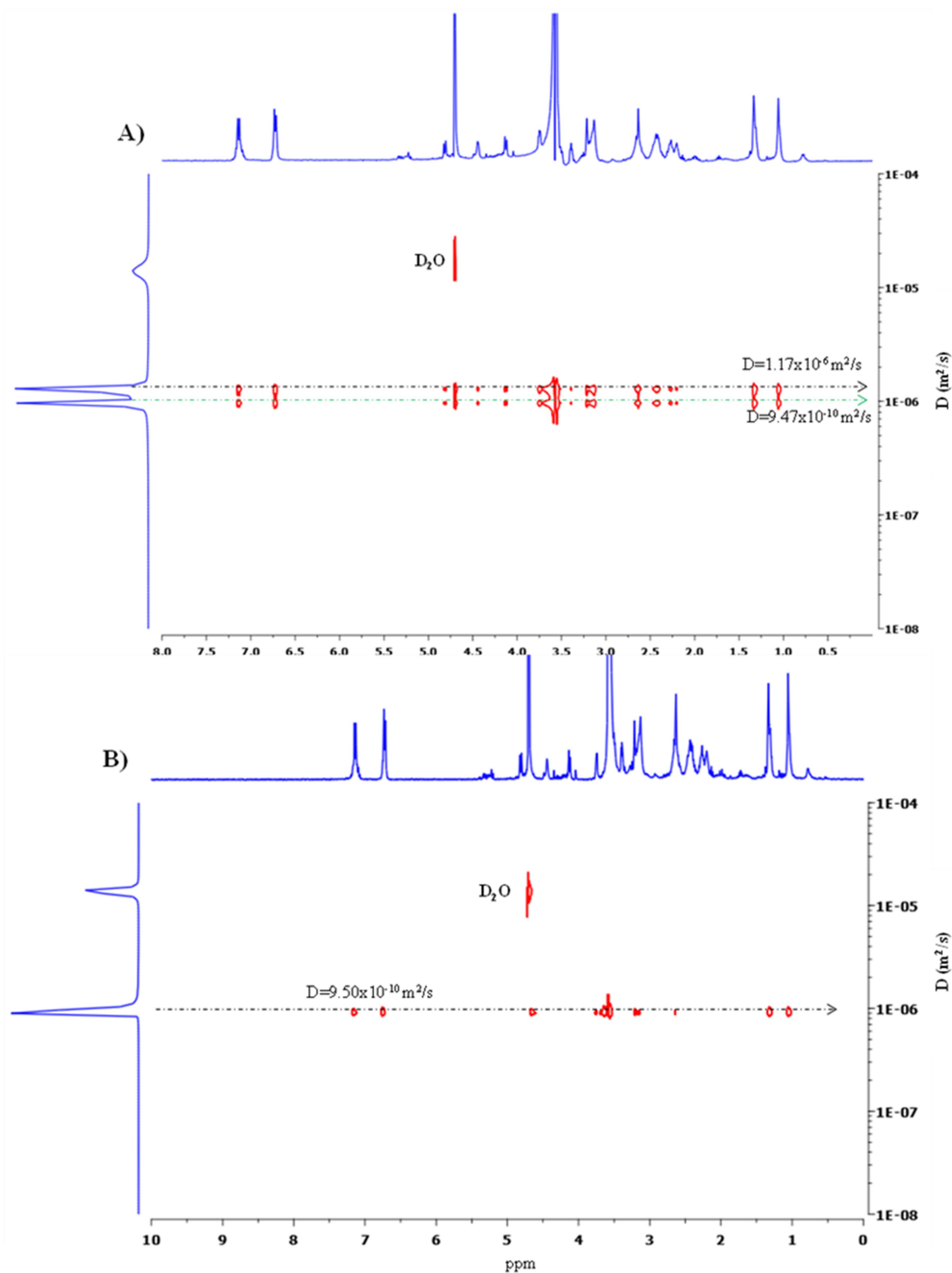


**Figure S2.** DOSY NMR spectra obtained at 300 K in D<sub>2</sub>O. Spectra of compound 49 before completion of the functionalization (A) and after completion of the functionalization (B). After incubation with more amount of AX, the two-translation diffusion (A) shift to one chemical shift and difusión behaviour (B).

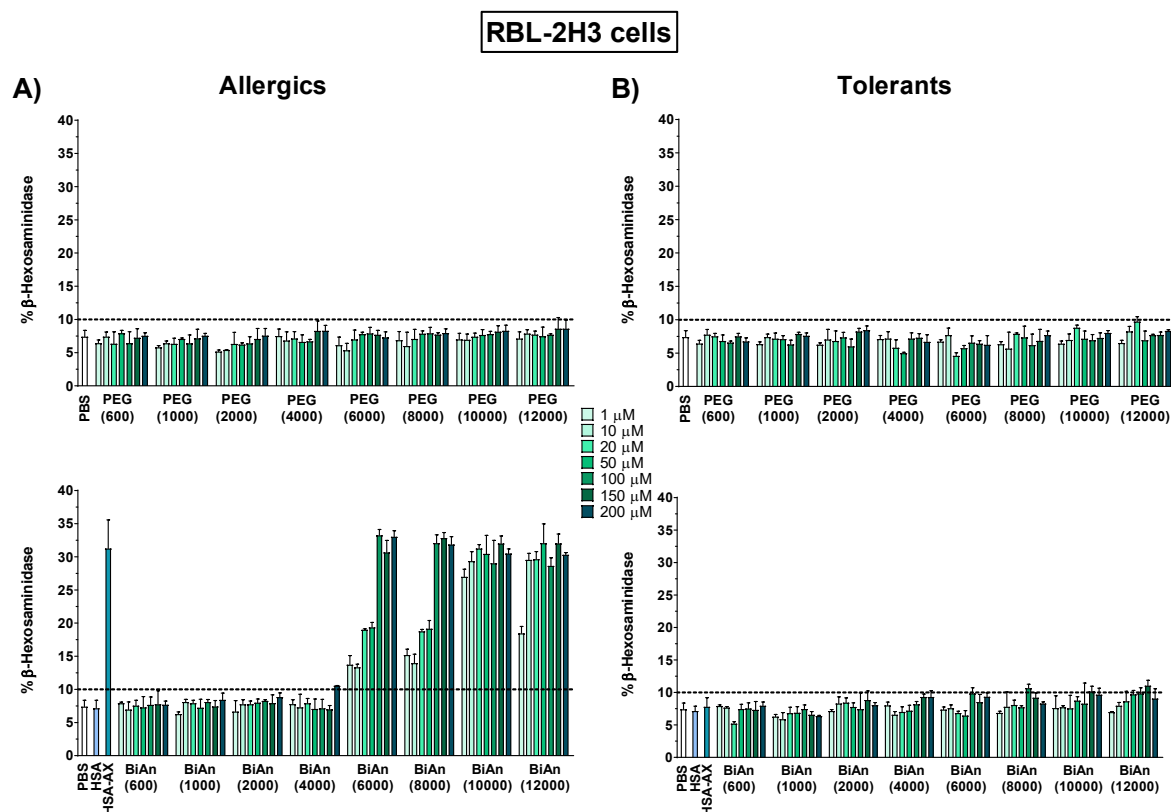


**Figure S3.** Impact of BiAns on RBL-2H3 degranulation at different concentrations (1  $\mu$ M, 10  $\mu$ M, 20  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M, 150  $\mu$ M, 200  $\mu$ M). **A)** Percentage of  $\beta$ -hexosaminidase release after incubation with PEG (without functionalization with AX) as negative controls (top) and BiAns (bottom) on sensitized cells with sera from allergic subjects. **B)** Percentage of  $\beta$ -hexosaminidase produced after incubation with PEG (without functionalization with AX) as negative controls (top) and BiAns (bottom) on sensitized cells with sera from tolerant subjects. HSA and HSA-AX were used as a negative and positive control, respectively. Data are expressed as means  $\pm$  S.D and are representative of three independent experiments. Groups with different letters statistically differ ( $P \leq 0.05$ ). All statistical analysis was performed using GraphPad Software Prism 8 (version 8.4.2). For multiple comparisons of quantitative data, one-way analysis of variance (ANOVA) was used followed by Tukey's *post-hoc* test.  $P < 0.05$  considered to indicate a statistically significant difference.

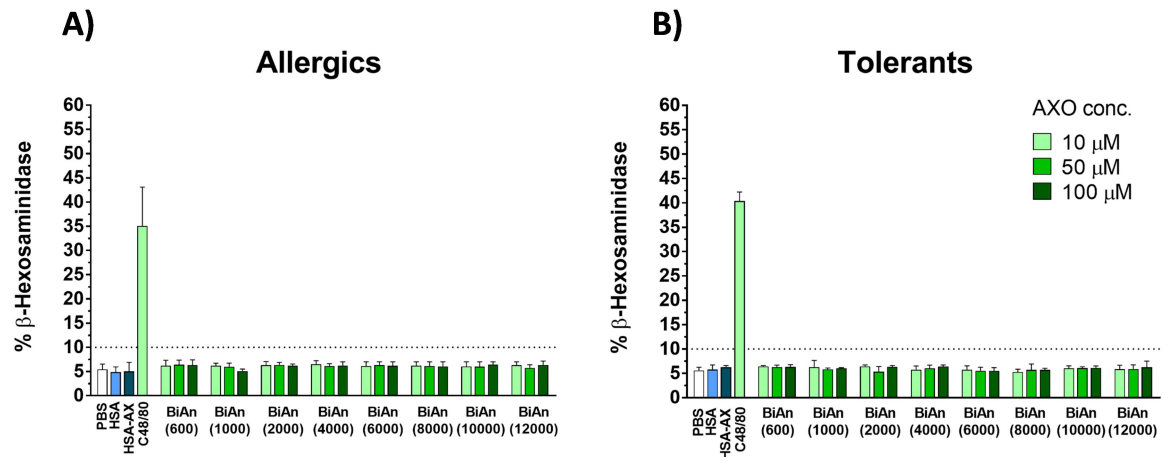


**Figure S4.** Impact of BiAns on HMC 1.2 degranulation at different concentrations (10  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M). These experiments were performed by sensitizing with serum from 3 tolerant and 6 allergic patients before the incubation with the BiAns, and then,  $\beta$ -hexosaminidase release was quantified. **A)** Percentage of  $\beta$ -hexosaminidase release after incubation with BiAns on sensitized cells with sera from allergic subjects. **B)** Percentage of  $\beta$ -hexosaminidase produced after incubation with BiAns on sensitized cells with sera from tolerant subjects. HSA and HSA-AX were used as negative controls and C48/80 as positive control. No statistically significant difference were found.

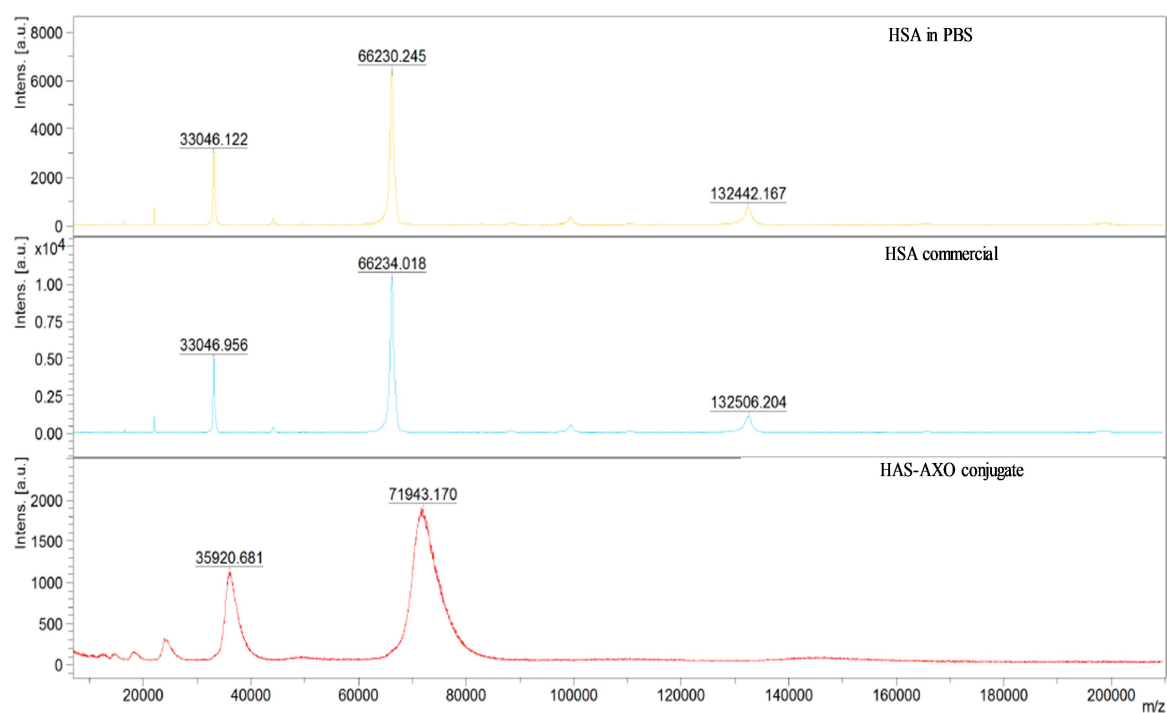


**Figure S5.** MALDI-TOF MS spectra of HSA control sample in PBS (yellow), commercial (blue) and HSA-AXO conjugate (red).

## **2. Supplementary Methods**

### **2.1. Purification of anti-AXO Monoclonal Antibodies**

After concentration of the hybridomae supernatants with Amicon ultra-4 3K centrifugal filter, the antibodies were purified by affinity chromatography: using Protein A/G agarose for AO3.2 (IgG<sub>2a</sub>) following the manufacturer guideline for NAb Protein A/G Spin Kit and a PLL-AXO coupled CNBr-activated sepharose 4B column for AO6.2 (IgE), as follows. The sepharose was activated by 1mM HCl at 4°C for 30 min and packed into a column. The column was conditioned with 5mL of borate-saline buffer (pH 8.3). The PLL-AXO conjugate in 5mL of borate-saline buffer (pH 8.3) was added to the column and incubated for 2 h at room temperature under agitation. The excess and uncoupled PLL-AXO was eliminated by adding 25mL of borate-saline buffer (pH 8.3). The unreacted group was blocked with 5mL of 50mM bicarbonate-ethanolamine buffer. The blocking solution was removed with an extensive wash, first with borate-saline buffer (pH 8.3), then with acetate buffer (0.1M, pH 4) containing 0.5M NaCl. These wash cycles of high and low pH buffer solutions were repeated five times. Then, the column was washed with PBS (25mL) and 25mL of Tris buffer (0.1M)-HCl (2M). The hybridoma supernatant was filtered with a 0.2µm filter. The filtered supernatant (60mL) was mixed with 4mL Tris buffer (1.5M, pH 8.8), 40mL Tris buffer (0.1M)-HCl (2M) pH 8.6, and 7.012g of NaCl. The resulting mixture was passed through the column. The first 2mL of the eluent was discarded as it might contain Tris buffer. The other portions of the collected fractions were re-eluted through the column. Another 25 mL of Tris buffer (0.1M)-HCl (2M) was passed through the column to remove the traces of the supernatant and excess antibody remained unattached. Finally, 10mL of diethylamine (50mM) was added to collect an eluent of 1mL volume in a vial containing 300µL of Tris buffer (1M, pH 6.8). The fractions containing the antibody were collected after measuring the absorbance at 280nm and dialysed against PBS at 4°C for 72 h (changing PBS every 24 h) to eliminate the diethylamine.

## 2.2. Sample Preparation for TEM and Micrograph Acquisition

The samples were prepared according to Optimized Negative-staining (OpNS)<sup>1</sup>. Briefly, 15 µg/mL of MoAb (AO3.2) at different molar ratios were incubated with BiAns for 90 min at room temperature. Aliquots (5 µL) of the samples adhered on carbon-film copper grids, which made hydrophilic by glow-discharge for 15 s, at 1 mbar. After incubating for 15 min at 4 °C, grids were dried on a filter paper to remove sample excess and washed with 30 µL of Milli-Q water for a few seconds. The water excess was dried, and samples were exposed to 15 µL of 1% (w/v) uranyl formate for 20-30 s protected from light at 4 °C. Finally, the uranyl formate solution excess was removed with filter paper and left to dry at room temperature. The acquisition of the images was performed using Talos F200X 200 kV D6368 XTwin electron microscope operating at 80 KV. Magnification was set at 450000X to obtain the data with STEM nP SA zoom diffraction mode. From the different tested MoAbs to BiAn ratios, a molar ratio of 8:1 was found to be the optimal condition. Images were processed using Image J software, determining the negatively-stained area of each complex (a minimum of 230 particles were measured per condition). Then, the number of MoAbs per complex was estimated by comparing the obtained area for each complex with the average area occupied by individual MoAbs not incubated with the nanostructures ( $57 \pm 12.7 \text{ nm}^2$ ).

## 2.3. Cell Viability Assays

The MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay was performed according to the manufacturer's instructions (CellTiter 96 RAQueous One Solution Cell Proliferation Assay, Promega, Wisconsin, USA). HumRBL-2H3 cells were obtained from the Cell Culture Unit of the University of Granada (Granada, Spain) and cultured in RPMI containing 10% heat-inactivated FBS, 2mM L-Glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin, in a humidified 5% CO<sub>2</sub> atmosphere at 37°C. HumRBL-2H3 cells were seeded into 96-well plates ( $2 \times 10^5$  cells/well) and incubated for 24h with different concentrations of BiAns (1 µM, 10 µM, 20 µM, 50 µM, 100 µM, 150 µM, 200 µM, in terms of AXO equivalents). Cells incubated only with the medium were included as a control group. Later, MTS was added to each well and incubated for 3h. The absorbance was measured at 490 nm. Cellular viability

was determined by comparing the absorbance values obtained for BiAns with those from untreated control cells.

## 2.4. Characterization Methods

$^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra were recorded in  $\text{D}_2\text{O}$  and  $\text{CDCl}_3$  on a Bruker Ascend<sup>TM</sup> 400 MHz spectrometer (Rheinstetten, Germany) equipped with a 5 mm BBFO<sup>PLUS</sup> probe with  $^2\text{H}$  "lock" channel and z gradient. All chemical shifts ( $\delta$ ) are reported in parts per million (ppm) using deuterated solvent resonance as an internal standard. The spectra were analyzed using Mestre Nova version 12.0.3-1284 from Mestrelab Research (Santiago de Compostela, Spain).

### 2.4.1. NMR Diffusion Experiments

The Diffusion-Ordered Spectroscopy (DOSY) experiments were performed on samples of 1mM concentration (within reported infinite dilution ranges of related samples at 0.1–2.1 mM) in  $\text{D}_2\text{O}$ . The spectra were recorded on a Bruker Ascend 400<sup>TM</sup> MHz spectrometer equipped with a temperature control unit ranging from  $-50$  to  $50^\circ\text{C}$ . The temperature was calibrated to 300K using  $\text{CD}_3\text{OD}$ . The gradient strength was set by measuring the diffusion rate of the residual HDO in  $\text{D}_2\text{O}$ . For all experiments,  $^1\text{H}$  NMR was recorded before the DOSY experiments. A total of 16 scans with 10 s relaxation delay were recorded by setting the gradient pulse duration to 3 ms and diffusion delay to 100 ms. The spectra were processed in the Mestre Nova DOSY package by baseline correction, phase correction, spectral alignment, and reference deconvulsion before Bayesian DOSY transformation. The diffusion coefficients ( $D$ ) of the nanostructures were obtained by fitting the signal decay into the Stejskal-Tanner equation<sup>2</sup>:  $\ln\left(\frac{I}{I_0}\right) = -\gamma^2 \delta^2 G^2 \left(\Delta - \frac{\sigma}{3}\right) D$ , where  $G$  is the gradient strength,  $I$  intensity at a given value  $G$ ,  $I_0$  is intensity at  $G = 0$ ,  $\gamma$  is gyromagnetic constant,  $\delta$  is gradient duration,  $\Delta$  is echo delay, and  $D$  is diffusion coefficient.

An example of the utility of this “NMR chromatography” is given (Figure S2) in detecting traces of impurities, incomplete haptenated bidendrons or starting materials, as they permit discriminating between the different component of a mixture by translational diffusion (depending on chemical shift and diffusion behaviour)<sup>3,4</sup>.

## 2.5. Synthetic Protocols

### 2.5.1. Preparation and characterization of HSA-AXO conjugate

HSA-AXO conjugate was prepared as previously described<sup>5</sup> by dissolving HSA (20 mg/mL) in PBS (1x, pH 7.4) and mixing with AX (200 mg/mL) in carbonate buffer (5mM, pH 10.2). The resulting mixture was incubated at 37°C for 48 h and purified by gel filtration chromatography using Sephadex G-10 (PD MidiTrap G-10). The HSA control sample was also prepared in the same conditions without AX. The product was characterized by a MALDI-TOF MS. Mass increment of 5712Da, which corresponds to approximately 14 AXO units, was observed for the modified HSA compared to the HSA control ([Figure S5](#)).

### 2.5.2. General Synthetic Protocols to obtain BiAns

Synthesis of PEG-(Ts)<sub>2</sub> (Procedure A). PEG (1 equiv.) dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> and 4-toluenesulfonyl chloride (TsCl) (3 equiv.) were placed in a 250 mL round-bottom flask, sparged with N<sub>2</sub>, and ice-cooled to 0°C. Powdered KOH (4 equiv.) was added in small portions in order to maintain the temperature of the mixture below 5 °C. After stirring for 5 h at 0 °C, the reaction took place at room temperature overnight. After a washing step with H<sub>2</sub>O, the organic layer was separated, and the aqueous phase was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3x15mL). The combined organic layer was dried over anhydrous MgSO<sub>4</sub>, filtered, and the solvent removed by a rotary evaporator to obtain a product.

**Synthesis of PEG-(N<sub>3</sub>)<sub>2</sub> (Procedure B).** PEG-(Ts)<sub>2</sub> (1 equiv.) was dissolved in 50 mL of H<sub>2</sub>O: THF (80:20), and NaN<sub>3</sub> (3 equiv.) was added to the dissolved product and refluxed at 70 °C overnight. After cooling the reaction to room temperature, the solvent was removed under reduced pressure and the dried product was dissolved in CH<sub>2</sub>Cl<sub>2</sub>. The product was cleaned by washing with H<sub>2</sub>O (3x30 mL), 1M HCl (10mL), and brine. The collected organic layer was dried over anhydrous MgSO<sub>4</sub>, filtered, and the solvent removed under reduced pressure to obtain a crude product.

**Synthesis of PEG-(NH<sub>2</sub>)<sub>2</sub> (Procedure C).** To a solution containing a weighed amount of PEG-(N<sub>3</sub>)<sub>2</sub> in 15 mL of MeOH, 20 wt. % of 10% Pd/C was added. The resulting suspension was stirred at 300 rpm under hydrogen gas (H<sub>2</sub>, 50 bar) at room temperature. The reaction was monitored by <sup>1</sup>H NMR by the presence of the peak pertaining to CH<sub>2</sub>-N<sub>3</sub>. When the reaction reached completion, the catalyst was removed by filtering through MeOH-washed Celite and the solvent was evaporated to yield the desired bisamine product.

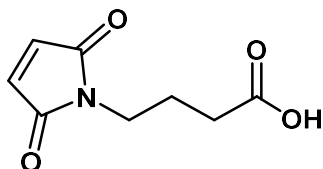
**Synthesis of PEG-bismaleimide (Procedure D).** To a stirred solution of compound 1 (2.2 equiv.) in anhydrous CH<sub>2</sub>Cl<sub>2</sub> at 0 °C, N-methyl morpholine (NMM) (2.3 equiv.) and isobutyl chloroformate (<sup>i</sup>BuCOCl) (2.3 equiv.) were sequentially added dropwise. After 1 h, PEG-(NH<sub>2</sub>)<sub>2</sub> (1 equiv.) dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (15 mL) was added. The reaction was slowly warmed to room temperature and run for 48 h. The product was washed with H<sub>2</sub>O, 5% HCl aqueous solution, H<sub>2</sub>O and 10% NaHCO<sub>3</sub> aqueous solution. The collected organic layer was dried over anhydrous MgSO<sub>4</sub>, filtered, and the solvent removed under reduced pressure. The crude product was purified by gel permeation chromatography using Bio-Beads™ S-X1 support (200-400 mesh, Bio-Rad) with THF as eluent.

**Synthesis of PEG-(dG<sub>2</sub>)<sub>2</sub> (Procedure E).** A solution of PolyAmidoAmine (PAMAM) dendrimer, cystamine core, generation 2 (G<sub>2</sub>) (1.1 equiv.) in PBS (1x, pH 6.5) was deoxygenated by purging with N<sub>2</sub> gas. Then tris (2-carboxyethyl) phosphine (TCEP) (10 equiv.) was added and the mixture was stirred. The reduction of the disulfide bond was monitored by TLC using Ellman's and ninhydrin as reagents. When reduction was completed, PEG-bismaleimide (1 equiv.) was dissolved in 5 mL of distilled H<sub>2</sub>O with few drops of DMSO as cosolvent and flushed with N<sub>2</sub> gas, added to the above solution, and the mixture was stirred overnight. The resulting residue was concentrated under reduced pressure, redissolved in distilled H<sub>2</sub>O for purifying the product by gel filtration chromatography using PD MidiTrap G-25.

**Synthesis of PEG-(dG<sub>2</sub>-(AXO)<sub>8</sub>)<sub>2</sub> (Procedure F).** An excess of AX (32 equiv.) was added to a solution of PEG-(dG<sub>2</sub>)<sub>2</sub> (1 equiv. of nanoarchitecture, 16 equiv. NH<sub>2</sub> group) in carbonate buffer (0.05M, pH 10.9), and the mixture was cooled at 4 °C under magnetic stirring for 7 days with refreshing on three different occasions with 20% of the initial mass of

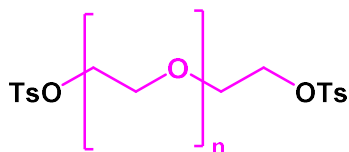
AX. The purification was performed by gel filtration chromatography using PD MidiTrap G-10 columns.

### Mal-COOH (1)



In a round bottom flask,  $\gamma$ -Aminobutyric acid (6 g, 58.2 mmol) and maleic anhydride (6.8 g, 69.9 mmol) were dissolved under string in 120 mL of glacial acetic acid at 120 °C for 6 hours. The reaction was cooled to room temperature before pouring into 50 mL of water and extracted with 50 mL of ethyl acetate on three occasions. The collected organic layers were combined and dried on anhydrous  $\text{MgSO}_4$ . The solvent was removed under reduced pressure by rotary evaporation. Purification was performed by recrystallization of the crude product four times in 1:1 hexane: ethyl acetate to obtain a product with a 65% yield.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$ : 10.81 (s, 1H), 6.71 (s, 2H), 3.60 (t,  $J$  = 6.8 Hz, 2H), 2.37 (t,  $J$  = 7.3 Hz, 2H), 1.93 (qt,  $J$  = 7.1 Hz, 2H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$ : 178.3, 170.9, 134.2, 37.1, 31.2, 23.6.

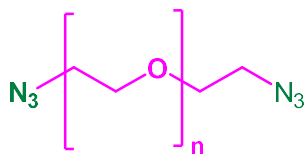
### PEG (600)- (Ts)<sub>2</sub> (2)



Synthesized according to general procedure A. PEG (MW~ 600 Da) (9 g, 15 mmol),  $\text{CH}_2\text{Cl}_2$  (30mL),  $\text{TsCl}$  (8.5 g, 45 mmol) and  $\text{KOH}$  (3.4 g, 60 mmol) were used in the reaction. The crude product was purified by column chromatography using  $\text{CH}_2\text{Cl}_2$ :  $\text{MeOH}$  (15:1 v/v) as an eluting solvent to obtain a white semisolid product with 93.3 % yield.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.78 (d,  $J$  = 8.3 Hz, 2H), 7.31 (d,  $J$  = 8.1 Hz, 2H), 4.12 (t, 4H), 3.67 – 3.55

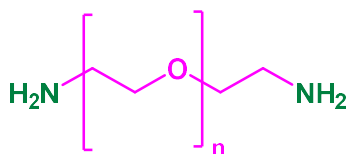
(m, 56H), 2.42 (s, 6H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  144.8, 133.0, 129.8, 128.0, 70.6, 69.3, 68.7, 21.7.

### PEG (600) -(N<sub>3</sub>)<sub>2</sub> (3)



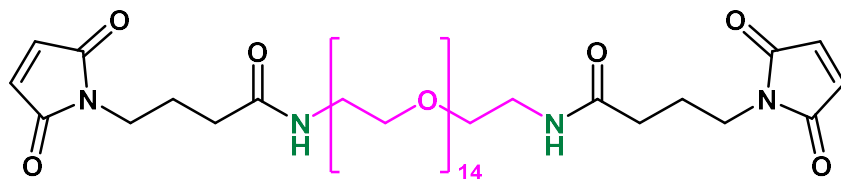
This was obtained according to general procedure B. **Compound 2** (8.4g, 9.24 mmol), 50 mL of distilled H<sub>2</sub>O: THF (80:20 %v/v) and NaN<sub>3</sub> (1.8 g, 27.6mmol) were used in the reaction. As a final point, the product was purified by silica gel column chromatography using CH<sub>2</sub>Cl<sub>2</sub>: MeOH (9:1 v/v) as eluent (84 % yield). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 3.82 – 3.37 (m, 56H), 3.32 (t, *J* = 5.1 Hz, 4H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 70.7, 70.0, 50.6.

### PEG (600) -(NH<sub>2</sub>)<sub>2</sub> (4)



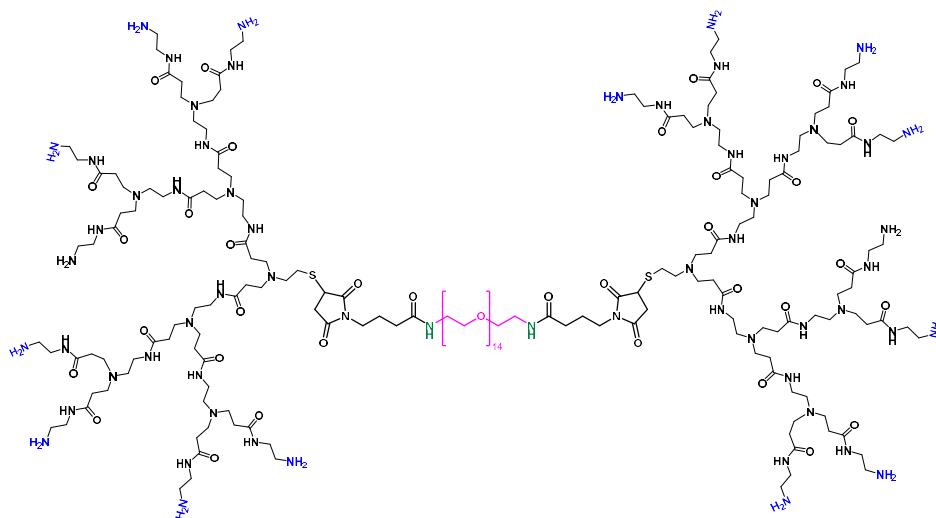
This was obtained according to general procedure C. **Compound 3** (500mg, 0.76 mmol), 15 mL of MeOH, Pd/C (100 mg) were used. The reaction was stirred for 6 h to obtain a white semisolid product with 84.5% of yield. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 3.67 – 3.57 (m, 56H), 3.48 (t, *J* = 5.2 Hz, 4H), 2.83 (t, *J* = 5.2 Hz, 4H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 73.3, 70.6, 70.3, 41.7.

### PEG (600) -(maleimide)<sub>2</sub> (5)



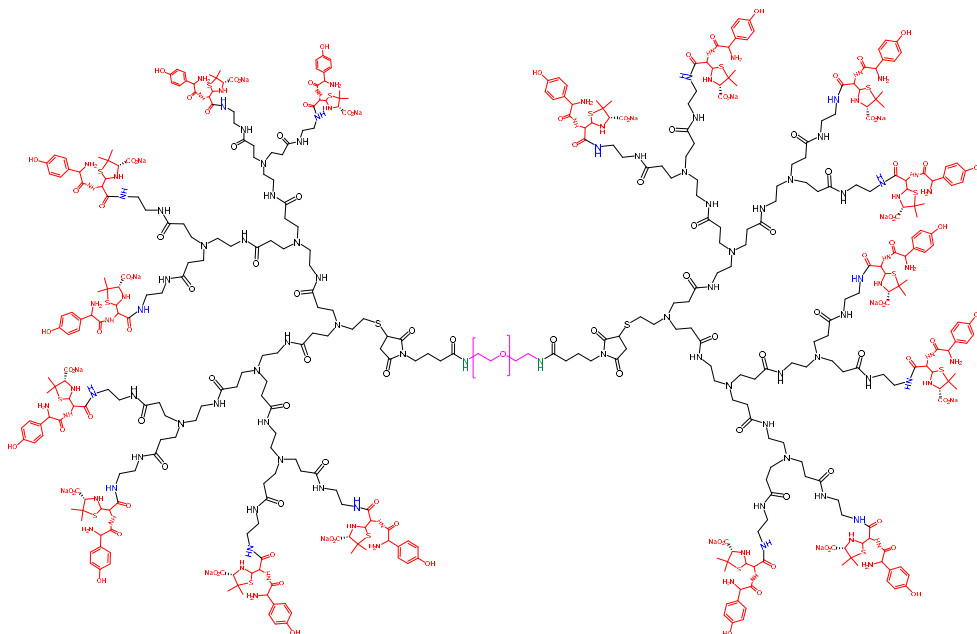
This is synthesized according to general procedure D. **Compound 1** (314 mg, 1.72 mmol), CH<sub>2</sub>Cl<sub>2</sub> (15 mL), NMM (200.74 mg, 1.79 mmol), <sup>i</sup>BuCoCl (234 mg, 1.79 mmol) and **compound 4** (466mg, 0.78 mmol) dissolved in 15 mL CH<sub>2</sub>Cl<sub>2</sub> were used to obtain a product with 60.4 % of yield. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 6.70 (s, 4H), 6.43 (s, 2H), 3.67 – 3.61 (m, 56H), 3.51–3.45 (m, 8H), 3.44 (t, *J* = 5.2 Hz, 4H), 2.17 (t, *J* = 7.2 Hz, 4H), 1.97 – 1.88 (m, 4H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 172.3, 171.2, 134.6, 70.9, 70.6, 70.3, 39.7, 37.7, 25.1.

## PEG (600)-[dG<sub>2</sub>]<sub>2</sub>(6)



This was synthesized according to general procedure E. G<sub>2</sub> (614 mg, 0.18mmol), 5 mL of PBS (1x, pH 6.5), TCEP (525mg, 1.83mmol), **compound 5** (150mg, 0.166 mmol), 5 mL of H<sub>2</sub>O and few drops of DMSO were used. The purification was performed gel filtration chromatography using PD MidiTrap G-10 columns and collecting the fractions of small volumes. <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O)  $\delta$  3.73 – 3.67 (m, 56H), 3.66– 3.59 (m, 8H), 3.55 – 3.26 (m, 88H), 3.15 (t,  $J$  = 6.0 Hz, 8H), 2.87 – 2.76 (m, 56H), 2.68 – 2.56 (m, 30H), 2.50 – 2.39 (m, 56H), 2.17 – 2.08 (m, 8H), 1.92 – 1.83 (m, 4H). <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O)  $\delta$  179.7, 178.7, 172.9, 172.6, 69.7, 69.6, 69.0, 51.9, 49.7, 49.5, 39.1, 37.0, 34.5, 30.2, 29.0, 28.4, 23.5, 22.9.

## PEG (600)-[dG<sub>2</sub>-(AXO)<sub>8</sub>]<sub>2</sub>(7): BiAn(600)



This was obtained by following general procedure F. **Compound 6** (80.5 mg, 0.021mmol) and AX (252.7mg, 0.65mmol) were reacted. The reaction was refreshed with 50.5 mg of AX on three different occasions. After purification the product was collected as yellowish powder with 59.4% of yield. <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O)  $\delta$  7.29 (d,  $J$  = 7.8 Hz, 32H), 6.87 (d,  $J$  = 7.8 Hz, 32H), 4.96 (d,  $J$  = 8.0 Hz, 16H), 4.59 (s, 16H), 4.27 (d,  $J$  = 8.0 Hz, 16H), 3.75 – 3.55 (m, 56H), 3.62 – 3.57(m,8H), 3.35(s,16H), 3.33 – 3.18 (m, 88H), 3.12 – 3.01 (m, 8H), 2.86 – 2.69 (m, 56H), 2.67 – 2.51 (m, 30H), 2.49 – 2.32 (m, 56H), 2.18– 2.05(m, 8H),1.97 – 1.75(m,4H), 1.47 (s, 48H), 1.20 (s, 48H). <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O)  $\delta$  175.6, 175.1, 174.6, 171.6, 160.5, 156.2, 129.7, 128.8, 115.9, 74.9, 69.8, 69.6, 68.9, 64.9, 59.5, 59.1, 58.2, 51.4, 49.1, 39.4, 38.9, 38.7, 36.9, 36.9, 3.7, 28.9, 28.2, 26.8, 23.6, 23.0.

## PEG (1k)- (Ts)<sub>2</sub> (8)

Synthesized according to general procedure A. PEG (MW~ 1000 Da) (10 g, 10 mmol), CH<sub>2</sub>Cl<sub>2</sub> (30mL), TsCl (5.7 g, 30 mmol) and KOH (2.2 g, 40 mmol) were used in the reaction. The crude product was purified by column chromatography using CH<sub>2</sub>Cl<sub>2</sub>: MeOH (9:1 v/v) as an eluting solvent to obtain a white powder product with 96.9 % yield. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  7.77 (d,  $J$  = 8.3 Hz, 2H), 7.32 (d,  $J$  = 8.1 Hz, 2H), 4.14 (t, 4H), 3.68 –

3.54 (m, 93H), 2.43(s, 6H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  144.7, 133.1, 129.7, 127.9, 70.6, 69.3, 68.7, 21.7.

#### PEG (1k) -(N<sub>3</sub>)<sub>2</sub> (9)

This was obtained according to general procedure B. **Compound 8** (8.7g, 6.65 mmol), 50 mL of distilled H<sub>2</sub>O: THF (80:20 %v/v) and NaN<sub>3</sub> (1.3 g, 19.95mmol) were used in the reaction. As a final point, the product was purified by silica gel column chromatography using 9:1(v/v) CH<sub>2</sub>Cl<sub>2</sub>: MeOH as an eluent to obtain a white powder of 93% of yield.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.64 – 3.56 (m, 93H), 3.34 (t,  $J$  = 5.1 Hz, 4H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  70.5, 70.0, 50.6.

#### PEG (1k) -(NH<sub>2</sub>)<sub>2</sub> (10)

This was obtained according to general procedure C. **Compound 9** (200 mg, 0.19 mmol), 15 mL of MeOH, Pd/C (40 mg) were used. The reaction took place for 12 h to obtain a product with 88 % yield.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.65 – 3.55 (m, 93H), 3.46 (t,  $J$  = 5.2 Hz, 4H), 2.81 (t,  $J$  = 5.2 Hz, 4H), 2.22(s, 4H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  73.3, 70.5, 70.2, 41.7.

#### PEG (1k) -(maleimide)<sub>2</sub> (11)

This was synthesized according to general procedure D. **Compound 1** (80.7 mg, 0.44 mmol), NMM (53.8 mg, 0.46 mmol),  $^i\text{BuCoCl}$  (62.8 mg, 0.46mmol.) and **compound 10** (200 mg, 0.2 mmol) and 15 mL CH<sub>2</sub>Cl<sub>2</sub> were used to obtain a product with 75.3 % of yield.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  6.69 (s, 4H), 6.52 (s, 2H), 3.68 – 3.59 (m, 93 H), 3.57-3.51 (m, 8H), 3.44 (t,  $J$  = 5.2 Hz, 4H), 2.17(t,  $J$  = 7.2 Hz, 4H), 1.93 – 1.88 (m, 4H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  172.0, 171.0, 134.2, 70.7, 70.6, 70.3, 39.4, 37.4, 24.8.

#### PEG (1k)-[dG<sub>2</sub>]<sub>2</sub> (12)

This was synthesized according to general procedure E. G<sub>2</sub> (100mg, 0.075mmol), 5 mL of PBS (1x, pH 6.5), TCEP (237.4mg, 1.83mmol), **Compound 11** (276 mg, 0.083 mmol) dissolved in 5 mL of H<sub>2</sub>O and few drops of DMSO. The purification was performed gel filtration chromatography using PD MidiTrap G-10 columns and collecting the fractions of small volumes. The product was isolated as white powder with 71.7% yield.  $^1\text{H}$  NMR (400 MHz, D<sub>2</sub>O)  $\delta$  3.75 – 3.68 (m, 93H), 3.67– 3.59 (m, 8H), 3.56 – 3.22 (m, 88H), 3.17 (t,  $J$  = 6.0

Hz, 8H), 2.87 – 2.74 (m, 56H), 2.73 – 2.55 (m, 30H), 2.52 – 2.41 (m, 56H), 2.22-2.08 (m, 8H), 1.92 – 1.83 (m, 4H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{D}_2\text{O}$ )  $\delta$  179.7, 179.5, 173.0, 172.7, 69.8, 69.6, 69.6, 51.9, 49.7, 49.4, 39.2, 38.9, 37.0, 34.5, 29.6, 29.1, 28.5, 23.5, 22.9.

#### PEG(1k)-[dG<sub>2</sub>-(AXO)<sub>8</sub>]<sub>2</sub> (13) : BiAn(1000)

This was obtained by following general procedure F. **Compound 12** (96.7 mg, 0.021mmol) and AX (256.3 mg, 0.65mmol) were reacted. The reaction was refreshed with 51 mg of AX on three different occasions. After purification the product was collected as yellowish powder with 75.6 % of yield.  $^1\text{H}$  NMR (400 MHz,  $\text{D}_2\text{O}$ )  $\delta$  7.29 (d,  $J$  = 7.8 Hz, 32H), 6.87 (d,  $J$  = 7.8 Hz, 32H), 4.96 (d,  $J$  = 8.0 Hz, 16H), 4.59 (s, 16H), 4.27 (d,  $J$  = 8.0 Hz, 16H), 3.75 – 3.55 (m, 93H), 3.62 – 3.57(m,8H), 3.35(s,16H), 3.33 – 3.18 (m, 88H), 3.12 – 3.01 (m, 8H), 2.86 – 2.69 (m, 56H), 2.67 – 2.51 (m, 30H), 2.49 – 2.32 (m, 56H), 2.18 – 2.05(m, 8H),1.84 – 1.72(m,4H), 1.47 (s, 48H), 1.20 (s, 48H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{D}_2\text{O}$ )  $\delta$  176.0, 175.4, 175.0, 172.0, 160.8, 156.6, 130.0, 129.1, 116.5, 75.1, 70.1, 69.9, 69.3, 65.2, 59.94, 59.4, 58.5, 51.7, 49.5, 39.8, 39.1, 37.2, 33.1, 29.2, 28.5, 27.2, 23.5, 23.4.

#### PEG (2k)- (Ts)<sub>2</sub> (14)

Synthesized according to general procedure A. PEG (MW~ 2000 Da) (10 g, 5 mmol),  $\text{CH}_2\text{Cl}_2$  (30mL), TsCl (2.8 g, 15 mmol) and KOH (2.24 g, 40 mmol) were used in the reaction. The crude product was purified by column chromatography using  $\text{CH}_2\text{Cl}_2$ : MeOH (9:1 v/v) as an eluting solvent to obtain a white solid product with 78 % yield.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.76 (d,  $J$  = 8.3 Hz, 2H), 7.33 (d,  $J$  = 8.1 Hz, 2H), 4.12 (t,  $J$  = 5.6 Hz, 4H), 3.67 – 3.54 (m, 184H), 2.42(s, 6H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  144.7, 133.1, 129.6, 128.0, 77.1, 70.3, 69.3, 68.7, 21.5.

#### PEG (2k) -(N<sub>3</sub>)<sub>2</sub> (15)

This was obtained according to general procedure B. **Compound 14** (10.7g, 4.6 mmol), 50 mL of distilled  $\text{H}_2\text{O}$ : THF (80:20 %v/v) and  $\text{NaN}_3$  (900 mg, 13.8 mmol) were used in the reaction. As a final point, the product was purified by silica gel column chromatography using 9:1(v/v)  $\text{CH}_2\text{Cl}_2$ : MeOH as an eluent to obtain a white powder with 93.1 % of yield.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.67 – 3.54 (m, 186H), 3.39 (t,  $J$  = 5.1 Hz, 4H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  70.6, 70.0, 50.7.



### PEG (2k) -(NH<sub>2</sub>)<sub>2</sub> (16)

This was obtained according to general procedure C. Compound 15 (200 mg, 0.097 mmol), 15 mL of MeOH, Pd/C (40 mg) were used. The reaction was stirred for 12 h to obtain a product with 89.2%. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 3.73 – 3.55 (m, 186H), 3.52 (t, *J* = 5.2 Hz, 4H), 2.88 (t, *J* = 5.2 Hz, 4H), 1.95(s, 4H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 73.6, 70.7, 70.4, 41.7.

### PEG (2k) -(maleimide)<sub>2</sub> (17)

This is synthesized according to general procedure D. **Compound 1** (101 mg, 0.56 mmol), NMM (151 mg, 1.3 mmol), <sup>t</sup>BuCoCl (176 mg, 1.3 mmol) and PEG (2000)-diamine (500 mg, 0.25 mmol) and 15 mL CH<sub>2</sub>Cl<sub>2</sub> were used to obtain a white solid product with 79.5 % of yield. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 6.70 (s, 4H), 6.39 (s, 2H), 3.70 – 3.59 (m, 186 H), 3.58-3.53 (m, 8H), 3.45 (t, *J* = 5.2 Hz, 4H), 2.16(t, *J* = 7.2 Hz, 4H), 1.98 – 1.90 (m, 4H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 172.0, 171.0, 134.2, 70.7, 70.6, 70.3, 39.4, 37.4, 24.8.

### PEG (2k)-[dG<sub>2</sub>]<sub>2</sub> (18)

This was synthesized according to general procedure E. G<sub>2</sub> (157mg, 0.047mmol), 5 mL of PBS (1x, pH 6.5), TCEP (135mg, 0.47mmol), **Compound 17** (100 mg, 0.043 mmol) dissolved in 5 mL of H<sub>2</sub>O and few drops of DMSO. The purification was performed gel filtration chromatography using PD MidiTrap G-25 columns. The product was isolated as white powder with 63.9 % yield. <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O) δ 3.77 – 3.66 (m, 186H), 3.62–3.48 (m, 8H), 3.46 – 3.19 (m, 88H), 3.17 (t, *J* = 6.0 Hz, 8H), 2.85 – 2.68 (m, 56H), 2.67 – 2.53 (m, 30H), 2.50 – 2.40 (m, 56H), 2.17-2.06 (m, 8H), 1.92 – 1.77 (m, 4H). <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O) δ 179.8, 178.6, 173.0, 172.8, 69.9, 69.7, 69.1, 52.1, 49.8, 49.6, 39.3, 39.0, 37.2, 34.7, 29.2, 28.6, 23.7, 23.0.

### PEG(2k)-[dG<sub>2</sub>-(AXO)<sub>8</sub>]<sub>2</sub> (19) : **BiAn(2000)**

This was obtained by following general procedure F. **Compound 18** (98.2 mg, 0.0173mmol) and AX (214.45mg, 0.554 mmol) were reacted. The reaction was refreshed with 43 mg of AX on three different occasions. After purification the product was collected as yellowish powder with 70.7 % of yield. <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O) δ 7.30 (d, *J* = 7.8 Hz, 32H), 6.88 (d, *J* = 7.8 Hz, 32H), 4.95 (d, *J* = 8.0 Hz, 16H), 4.67 (s, 16H), 4.27 (d, *J* = 8.0 Hz,

16H), 3.89 – 3.56 (m, 186H), 3.54 – 3.43(m,8H), 3.35(s,16H), 3.33 – 3.20 (m, 88H), 3.16 – 3.11 (m, 8H), 2.90 – 2.69 (m, 56H), 2.68 – 2.53 (m, 30H), 2.50 – 2.29 (m, 56H), 2.27 – 2.09 (m, 8H), 1.92 – 1.71(m,4H), 1.46 (s, 48H), 1.20 (s, 48H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{D}_2\text{O}$ )  $\delta$  175.7, 175.1, 174.9, 171.6, 160.4, 156.3, 129.7, 129.0, 116.2, 74.9, 69.8, 69.6, 69.0, 64.9, 59.6, 59.1, 57.9, 51.4, 49.1, 39.4, 38.7, 37.3, 32.7, 28.9, 28.2, 26.8, 23.7, 23.1.

#### PEG (4k) -(Ts)<sub>2</sub> (20)

Synthesized according to general procedure A. PEG (MW~ 4000 Da) (10 g, 2.5 mmol),  $\text{CH}_2\text{Cl}_2$  (30mL), TsCl (1.43 g, 7.5 mmol) and KOH (1.68 g, 3 mmol) were used in the reaction. The crude product was purified by column chromatography using  $\text{CH}_2\text{Cl}_2$ : MeOH (9.5:0.5 v/v) as an eluting solvent to obtain a white powder product with 84.4 % yield.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.77 (d,  $J$  = 8.3 Hz, 2H), 7.34 (d,  $J$  = 8.1 Hz, 2H), 4.14 (t, 4H), 3.68 – 3.54 (m, 373H), 2.43(s, 6H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  144.8, 132.9, 129.6, 128.0, 77.1, 70.6, 69.3, 68.7, 21.5.

#### PEG (4k) -(N<sub>3</sub>)<sub>2</sub> (21)

This was obtained according to general procedure B. **Compound 20** (9.1g, 2.1 mmol), 50 mL of distilled  $\text{H}_2\text{O}$ : THF (80:20 %v/v) and  $\text{NaN}_3$  (0.41 g, 6.34 mmol) were used in the reaction. As a final point, the product was purified by silica gel column chromatography using 9:1(v/v)  $\text{CH}_2\text{Cl}_2$ : MeOH as an eluent to obtain a white powder of 90.5% of yield.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.85 – 3.44 (m, 373H), 3.38 (t,  $J$  = 5.1 Hz, 4H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  70.6, 70.2, 50.8.

#### PEG (4k) -(NH<sub>2</sub>)<sub>2</sub> (22)

This was obtained according to general procedure C. **Compound 21** (200 mg, 0.049 mmol), 15 mL of MeOH, Pd/C (40 mg) were used. The reaction was stirred for 12 h to obtain product with 96.7 % yield.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.73 – 3.54 (m, 373H), 3.51 (t,  $J$  = 5.2 Hz, 4H), 2.86 (t,  $J$  = 5.2 Hz, 4H), 1.74(s, 4H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  73.6, 70.7, 70.4, 41.9.

#### PEG (4k) -(maleimide)<sub>2</sub> (23)

This is synthesized according to general procedure D. **Compound 1** (46.8mg, 0.256 mmol), NMM (61.6 mg, 0.526 mmol),  $i\text{BuCoCl}$  (71.8 mg, 0.526 mmol.) and **Compound 22**

(500 mg, 0.125 mmol) and 15 mL CH<sub>2</sub>Cl<sub>2</sub> were used to obtain a product with 75.9 % of yield. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 6.70 (s, 4H), 6.50 (s, 2H), 3.68 – 3.60 (m, 373 H), 3.59–3.53 (m, 8H), 3.45 (t, *J* = 5.2 Hz, 4H), 2.35(t, *J* = 7.2 Hz, 4H), 1.97 – 1.86 (m, 4H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 172.0, 171.3, 134.2, 70.7, 70.3, 70.0, 39.3, 37.5, 33.63, 24.5.

#### PEG (4k)-[dG<sub>2</sub>]<sub>2</sub> (24)

This was synthesized according to general procedure E. G<sub>2</sub> (84.7mg, 0.0253mmol), 5 mL of PBS (1x, pH 6.5), TCEP (72.5mg, 0.253mmol), **Compound 23** (100mg, 0.023 mmol) dissolved in 5 mL of H<sub>2</sub>O and few drops of DMSO. The purification was performed gel filtration chromatography using PD MidiTrap G-25columns. The product was isolated as white powder with 83.3% yield. <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O) δ 3.83 – 3.68 (m, 373 H), 3.68–3.59 (m, 8H), 3.58 – 3.22 (m, 88H), 3.17 (t, *J* = 6.0 Hz, 8H), 2.92 – 2.79 (m, 56H), 2.78 – 2.59 (m, 30H), 2.58 – 2.44 (m, 56H), 2.22– 2.11 (m, 8H), 1.91 – 1.80 (m, 4H). <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O) δ 178.5, 178.4, 172.9, 172.5, 69.9, 69.7, 69.1, 52.1, 49.9, 49.6, 39.3, 39.0, 37.1, 34.6, 29.7, 29.0, 27.6, 23.2, 22.6.

#### PEG(4k)-[dG<sub>2</sub>-(AXO)<sub>8</sub>]<sub>2</sub> (25) : **BiAn(4000)**

This was obtained by following general procedure F. **Compound 24** (93.5 mg, 0.0122mmol) and AX (151mg, 0.39mmol) were reacted. The reaction was refreshed with 30 mg of AX on three different occasions. After purification the product was collected as yellowish powder with 56.8 % of yield. <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O) δ 7.30 (d, *J* = 7.8 Hz, 32H), 6.87 (d, *J* = 7.8 Hz, 32H), 4.96 (d, *J* = 8.0 Hz, 16H), 4.59 (s, 16H), 4.27 (d, *J* = 8.0 Hz, 16H), 3.85 – 3.62 (m, 373H), 3.57 – 3.49(m,8H), 3.36(s,16H), 3.34 – 3.12 (m, 88H), 3.11 – 3.01 (m, 8H), 2.88 – 2.67 (m, 56H), 2.66 – 2.49 (m, 30H), 2.47 – 2.29 (m, 56H), 2.18 – 2.08(m, 8H), 1.91 – 1.73(m,4H), 1.48 (s, 48H), 1.21 (s, 48H). <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O) δ 174.3, 174.2, 173.9, 170.9, 160.1, 155.6, 129.1, 128.2, 115.5, 74.0, 69.0, 68.9, 68.7, 64.1, 58.8, 58.3, 57.5, 51.6, 48.2, 38.2, 38.0, 36.2, 32.0, 28.1, 27.53, 26.1, 23.05, 22.4.

#### PEG (6k) -(Ts)<sub>2</sub> (26)

Synthesized according to general procedure A. PEG (MW~ 6000 Da) (10 g, 1.67 mmol), CH<sub>2</sub>Cl<sub>2</sub> (30mL), TsCl (960 mg, 5 mmol) and KOH (840 mg, 15 mmol) were used in the reaction. The crude product was purified by column chromatography using CH<sub>2</sub>Cl<sub>2</sub>:

MeOH (9.5:0.5 v/v) as an eluting solvent to obtain a white solid product with 81.6 % yield.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.79 (d,  $J$  = 8.3 Hz, 2H), 7.34 (d,  $J$  = 8.1 Hz, 2H), 4.14 (t,  $J$  = 5.6 Hz, 4H), 3.68 – 3.52 (m, 560H), 2.42(s, 6H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  144.7, 133.1, 129.9, 128.1, 70.6, 69.35, 68.7, 21.6.

#### PEG (6k) -(N<sub>3</sub>)<sub>2</sub> (27)

This was obtained according to general procedure B. **Compound 26** (8.5g, 1.35mmol), 50 mL of distilled H<sub>2</sub>O: THF (80:20 %v/v) and NaN<sub>3</sub> (263mg, 4.04 mmol) were used in the reaction. As a final point, the product was purified by silica gel column chromatography using 9.5:0.5(v/v) CH<sub>2</sub>Cl<sub>2</sub>: MeOH as an eluent to obtain a white powder with 95.5 % of yield.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.69 – 3.49 (m, 560H), 3.34 (t,  $J$  = 5.1 Hz, 4H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  70.5, 70.0, 50.5.

#### PEG (6k) -(NH<sub>2</sub>)<sub>2</sub> (28)

This was obtained according to general procedure C. **Compound 27** (200 mg, 0.033mmol), 15 mL of MeOH, Pd/C (40 mg) were used. The reaction was stirred for 24 h to obtain a product with 96.3%.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.72 – 3.59 (m, 560H), 3.48 (t,  $J$  = 5.2 Hz, 4H), 2.88 (t,  $J$  = 5.2 Hz, 4H), 1.92(s, 4H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  73.5, 70.5, 70.3, 41.6.

#### PEG (6k) -(maleimide)<sub>2</sub> (29)

This is synthesized according to general procedure D. **Compound 1** (33.6 mg, 0.183 mmol), NMM (49.3 mg, 0.421 mmol), <sup>i</sup>BuCoCl (57.5 mg, 0.421 mmol.) and **Compound 29** (500 mg, 0.083 mmol) and 15 mL CH<sub>2</sub>Cl<sub>2</sub> were used to obtain a white solid product with 82.3 % of yield.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  6.69 (s, 4H), 6.39 (s, 2H), 3.79 – 3.54 (m, 560 H), 3.61-3.53 (m, 8H), 3.47 (t,  $J$  = 5.2 Hz, 4H), 2.33(t,  $J$  = 7.2 Hz, 4H), 1.98 – 1.88 (m, 4H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  172.2, 170.9, 134.0, 70.7, 70.3, 69.9, 39.1, 37.2, 33.6, 24.7.

#### PEG (6k)-[dG<sub>2</sub>]<sub>2</sub> (30)

This was synthesized according to general procedure E. G<sub>2</sub> (58mg, 0.017mmol), 5 mL of PBS (1x, pH 6.5), TCEP (45.3mg, 0.158mmol), **Compound 29** (100 mg, 0.016 mmol) dissolved in 5 mL of H<sub>2</sub>O and few drops of DMSO. The purification was performed gel filtration chromatography using PD MidiTrap G-25 columns. The product was isolated as

white powder with 65.8 % yield.  $^1\text{H}$  NMR (400 MHz,  $\text{D}_2\text{O}$ )  $\delta$  3.74 – 3.69 (m, 560H), 3.68–3.60 (m, 8H), 3.57–3.28 (m, 88H), 3.18 (t,  $J$  = 6.0 Hz, 8H), 2.91 – 2.80 (m, 56H), 2.79 – 2.70 (m, 30H), 2.60 – 2.47 (m, 56H), 2.22–2.13 (m, 8H), 1.93 – 1.80 (m, 4H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{D}_2\text{O}$ )  $\delta$  178.5, 177.6, 173.0, 172.6, 69.9, 69.8, 69.2, 52.0, 49.7, 49.6, 39.3, 39.0, 37.2, 34.6, 29.4, 29.4, 29.1, 23.2, 22.5.

#### PEG(6k)-[dG<sub>2</sub>-(AXO)<sub>8</sub>]<sub>2</sub> (31) : BiAn(6000)

This was obtained by following general procedure F. **Compound 30** (100 mg, 0.0103mmol) and AX (128 mg, 0.554 mmol) were reacted. The reaction was refreshed with 25.6 mg of AX on three different occasions. After purification the product was collected as yellowish powder with 48.4 % of yield.  $^1\text{H}$  NMR (400 MHz,  $\text{D}_2\text{O}$ )  $\delta$  7.27 (d,  $J$  = 7.8 Hz, 32H), 6.86 (d,  $J$  = 7.8 Hz, 32H), 4.97 (d,  $J$  = 8.0 Hz, 16H), 4.59 (s, 16H), 4.26 (d,  $J$  = 8.0 Hz, 16H), 3.79 – 3.67 (m, 560H), 3.56 – 3.50(m,8H), 3.35(s,16H), 3.33 – 3.12 (m, 88H), 3.10 – 2.99 (m, 8H), 2.90 – 2.70 (m, 56H), 2.66 – 2.49 (m, 30H), 2.45 – 2.27 (m, 56H), 2.18 –2.07 (m, 8H),1.97– 1.70(m,4H), 1.47 (s, 48H), 1.20 (s, 48H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{D}_2\text{O}$ )  $\delta$  175.2, 174.7, 171.8, 161.0, 156.5, 129.8, 128.9, 116.3, 74.9, 69.9, 69.8, 65.0, 59.7, 59.2, 58.4, 51.6, 49.3, 38.8, 37.0, 33.0, 29.1, 27.9, 27.0, 23.9, 23.0.

#### PEG (8k) -(Ts)<sub>2</sub> (32)

Synthesized according to general procedure A. PEG (MW~ 8000 Da) (10 g, 1.25 mmol),  $\text{CH}_2\text{Cl}_2$  (30mL), TsCl (710 mg, 3.75mmol) and KOH (210 mg, 3.75 mmol) were used in the reaction. The crude product was purified by column chromatography using  $\text{CH}_2\text{Cl}_2$ : MeOH (7:1 v/v) as an eluting solvent to obtain a white solid product with 85.6 % yield.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.76 (d,  $J$  = 8.3 Hz, 2H), 7.33 (d,  $J$  = 8.1 Hz, 2H), 4.14 (t,  $J$  = 5.6 Hz, 4H), 3.76 – 3.51 (m, 746H), 2.42(s, 6H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  144.6, 133.0, 129.8, 127.8, 70.6, 69.2, 68.7, 21.7.

#### PEG (8k) -(N<sub>3</sub>)<sub>2</sub> (33)

This was obtained according to general procedure B. **Compound 32** (8.8g, 1.06 mmol), 50 mL of distilled  $\text{H}_2\text{O}$ : THF (80:20 %v/v) and  $\text{NaN}_3$  (210 mg, 3.18 mmol) were used in the reaction. As a final point, the product was purified by silica gel column chromatography using (7:1 v/v)  $\text{CH}_2\text{Cl}_2$ : MeOH as an eluent to obtain a white powder with

89.1 % of yield.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.68 – 3.44 (m, 746H), 3.27 (t,  $J$  = 5.1 Hz, 4H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  70.4, 69.9, 50.5.

#### PEG (8k) -(NH<sub>2</sub>)<sub>2</sub> (34)

This was obtained according to general procedure C. **Compound 33** (200 mg, 0.025mmol), 15 mL of MeOH, Pd/C (40 mg) were used. The reaction was stirred for 72 h to obtain a product with 85%.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.78 – 3.57 (m, 746H), 3.46 (t,  $J$  = 5.2 Hz, 4H), 2.87 (t,  $J$  = 5.2 Hz, 4H), 1.83(s, 4H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  73.6, 70.6, 69.9, 41.7.

#### PEG (8k) -(maleimide)<sub>2</sub> (35)

This is synthesized according to general procedure D. **Compound 1** (5 mg, 0.0275 mmol), NMM (7.2 mg, 0.0616 mmol),  $^i\text{BuCoCl}$  (8.4 mg, 0.0616 mmol.) and **Compound 34** (100 mg, 0.0125mmol) and 15 mL  $\text{CH}_2\text{Cl}_2$  were used to obtain a white solid product with 68.3 % of yield.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  6.70 (s, 4H), 6.32 (s, 2H), 3.80 – 3.52 (m, 746H), 3.62-3.53 (m, 8H), 3.48 (t,  $J$  = 5.2 Hz, 4H), 2.33(t,  $J$  = 7.2 Hz, 4H), 1.98 – 1.83 (m, 4H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  171.8, 170.9, 134.2, 70.6, 70.3, 70.0, 39.1, 37.2, 33.4, 24.7.

#### PEG (8k)-[dG<sub>2</sub>]<sub>2</sub> (36)

This was synthesized according to general procedure E. G<sub>2</sub> (31mg, 0.0092mmol), 5 mL of PBS (1x, pH 6.5), TCEP (24mg, 0.084mmol), **Compound 35** (70 mg, 0.0084 mmol) dissolved in 5 mL of H<sub>2</sub>O and few drops of DMSO. The purification was performed gel filtration chromatography using PD MidiTrap G-25 columns. The product was isolated as white powder with 57 % yield.  $^1\text{H}$  NMR (400 MHz, D<sub>2</sub>O)  $\delta$  3.75 – 3.69 (m, 746H), 3.68– 3.61 (m, 8H), 3.59– 3.25 (m, 88H), 3.18 (t,  $J$  = 6.0 Hz, 8H), 2.92 – 2.80 (m, 56H), 2.79 – 2.69 (m, 30H), 2.61 – 2.47 (m, 56H), 2.23– 2.14 (m, 8H), 1.96 – 1.81 (m, 4H).  $^{13}\text{C}$  NMR (101 MHz, D<sub>2</sub>O)  $\delta$  178.3, 177.5, 172.6, 172.2, 69.7, 69.6, 69.0, 51.8, 49.7, 49.4, 39.1, 38.8, 36.9, 34.4, 29.2, 29.2, 28.9, 23.0, 22.3, 15.5, 15.0.

#### PEG(8k)-[dG<sub>2</sub>-(AXO)<sub>8</sub>]<sub>2</sub> (37) : **BiAn(8000)**

This was obtained by following general procedure F. **Compound 36** (47 mg, 0.004mmol) and AX (50 mg, 0.129 mmol) were reacted. The reaction was refreshed with 10

mg of AX on three different occasions. After purification the product was collected as yellowish powder with 45.4 % of yield.  $^1\text{H}$  NMR (400 MHz,  $\text{D}_2\text{O}$ )  $\delta$  7.31 (d,  $J$  = 7.8 Hz, 32H), 6.87 (d,  $J$  = 7.8 Hz, 32H), 4.94 (d,  $J$  = 8.0 Hz, 16H), 4.62 (s, 16H), 4.29 (d,  $J$  = 8.0 Hz, 16H), 3.80 – 3.61 (m, 746H), 3.56 – 3.44 (m, 8H), 3.34 (s, 16H), 3.32 – 3.05 (m, 88H), 3.14 – 3.02 (m, 8H), 2.90 – 2.71 (m, 56H), 2.68 – 2.50 (m, 30H), 2.47 – 2.26 (m, 56H), 2.18 – 2.08 (m, 8H), 1.90 – 1.72 (m, 4H), 1.46 (s, 48H), 1.19 (s, 48H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{D}_2\text{O}$ )  $\delta$  175.1, 175.0, 174.6, 171.6, 160.9, 156.3, 129.7, 128.8, 116.2, 74.8, 69.8, 69.6, 69.6, 64.9, 59.5, 59.0, 58.2, 51.3, 49.1, 39.4, 38.8, 36.9, 32.8, 28.9, 26.8, 23.7, 23.1.

### PEG (10k) -(Ts)<sub>2</sub> (38)

Synthesized according to general procedure A. PEG (MW~ 10000 Da) (10 g, 1 mmol),  $\text{CH}_2\text{Cl}_2$  (30 mL), TsCl (570 mg, 3 mmol) and KOH (168 mg, 3 mmol) were used in the reaction. The crude product was purified by column chromatography using  $\text{CH}_2\text{Cl}_2$ : MeOH (6:1 v/v) as an eluting solvent to obtain a white solid product with 70.8 % yield.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.80 (d,  $J$  = 8.3 Hz, 2H), 7.32 (d,  $J$  = 8.1 Hz, 2H), 4.14 (t,  $J$  = 5.6 Hz, 4H), 3.74 – 3.45 (m, 933H), 2.44 (s, 6H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  144.9, 133.1, 129.9, 128.1, 70.6, 69.3, 68.7, 21.8.

### PEG (10k) -(N<sub>3</sub>)<sub>2</sub> (39)

This was obtained according to general procedure B. **Compound 38** (7.2 g, 0.7 mmol), 50 mL of distilled  $\text{H}_2\text{O}$ : THF (80:20 %v/v) and  $\text{NaN}_3$  (136 mg, 2.1 mmol) were used in the reaction. As a final point, the product was purified by silica gel column chromatography using (6:1 v/v)  $\text{CH}_2\text{Cl}_2$ : MeOH as an eluent to obtain a white powder with 89.6 % of yield.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.62 – 3.41 (m, 933H), 3.27 (t,  $J$  = 5.1 Hz, 4H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  70.2, 69.7, 50.3.

### PEG (10k) -(NH<sub>2</sub>)<sub>2</sub> (40)

This was obtained according to general procedure C. **Compound 39** (100 mg, 0.00995 mmol), 15 mL of MeOH, Pd/C (20 mg) were used. The reaction was stirred for 96 h to obtain a product with 84.4%.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  3.74 – 3.58 (m, 933H), 3.45 (t,  $J$  = 5.2 Hz, 4H), 2.87 (t,  $J$  = 5.2 Hz, 4H), 1.78 (s, 4H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  73.6, 70.6, 69.9, 41.9.

### PEG (10k) -(maleimide)<sub>2</sub> (41)

This is synthesized according to general procedure D. **Compound 1** (3.8 mg, 0.021 mmol), NMM (5.6 mg, 0.0475 mmol), <sup>t</sup>BuCoCl (6.5mg, 0.0475 mmol) and **Compound 40** (94 mg, 0.0094mmol) and 15 mL CH<sub>2</sub>Cl<sub>2</sub> were used to obtain a white solid product with 54.6% of yield. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 6.70 (s, 4H), 6.32 (s, 2H), 3.79 – 3.61 (m, 933H), 3.62-3.53 (m,8H), 3.47 (t, *J* = 5.2 Hz, 4H), 2.33(t, *J* = 7.2 Hz, 4H), 1.99 – 1.87 (m, 4H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 172.01, 170.9, 134.2,70.7, 70.3, 70.0, 39.3, 37.2, 33.4, 24.9.

### PEG (10k)-[dG<sub>2</sub>]<sub>2</sub> (42)

This was synthesized according to general procedure E. G<sub>2</sub> (18mg, 0.0055mmol), 5 mL of PBS (1x, pH 6.5), TCEP (15.8mg, 0.055mmol), **Compound 41** (52 mg, 0.0084 mmol) dissolved in 5 mL of H<sub>2</sub>O and few drops of DMSO. The purification was performed gel filtration chromatography using PD MidiTrap G-25 columns. The product was isolated as white powder with 59.6 % yield. <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O) δ 3.74 – 3.69 (m, 993H), 3.68– 3.61 (m, 8H), 3.58– 3.28 (m, 88H), 3.18 (t, *J* = 6.0 Hz, 8H), 2.91 – 2.82 (m, 56H), 2.80 – 2.69 (m, 30H), 2.61 – 2.46 (m, 56H), 2.25– 2.14 (m, 8H), 1.93 – 1.81 (m, 4H). <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O) δ 178.2, 178.1, 172.8, 172.5, 69.8, 69.8, 69.5, 51.9, 49.7, 49.6, 39.1, 39.1, 38.9, 38.9, 34.4, 29.2, 29.2, 28.9, 23.03, 22.3.

### PEG (10k)-[dG<sub>2</sub>-(AXO)<sub>8</sub>]<sub>2</sub> (43) : BiAn(10000)

This was obtained by following general procedure F. **Compound 42** (40 mg, 0.0029mmol) and AX (36.3 mg, 0.095 mmol) were reacted. The reaction was refreshed with 7mg of AX on three different occasions. After purification the product was collected as yellowish powder with 45.4 % of yield. <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O) δ 7.29 (d, *J* = 7.8 Hz, 32H), 6.89 (d, *J* = 7.8 Hz, 32H), 4.94 (d, *J* = 8.0 Hz, 16H), 4.63 (s, 16H), 4.29(d, *J* = 8.0 Hz, 16H), 3.88 – 3.57 (m, 993H), 3.56 – 3.51(m,8H), 3.34(s,16H), 3.32 – 3.05 (m, 88H), 3.12 – 3.03 (m, 8H), 2.89 – 2.69 (m, 56H), 2.66 – 2.51 (m, 30H), 2.48 – 2.26 (m, 56H), 2.18 –2.06 (m, 8H),1.90– 1.77 (m,4H), 1.46 (s, 48H), 1.19 (s, 48H). <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O) δ 175.1, 174.6, 171.6, 160.7, 156.3, 129.7, 128.9, 116.2, 74.8, 69.8, 64.9, 59.6, 59.0, 58.1, 51.4, 49.3, 49.2, 39.4, 38.6, 36.9, 32.9, 28.9, 26.8, 26.7, 23.99, 23.0.

### PEG (12k) -(Ts)<sub>2</sub> (44)

Synthesized according to general procedure A. PEG (MW~ 12000 Da) (10 g, 0.83 mmol), CH<sub>2</sub>Cl<sub>2</sub> (30mL), TsCl (480 mg, 2.5mmol) and KOH (140 mg, 2.5mmol) were used in the reaction. The crude product was purified by column chromatography using CH<sub>2</sub>Cl<sub>2</sub>: MeOH (5:1 v/v) as an eluting solvent to obtain a white solid product with 70.5 % yield. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.79 (d, *J* = 8.3 Hz, 2H), 7.32 (d, *J* = 8.1 Hz, 2H), 4.14 (t, *J* = 5.6 Hz, 4H), 3.78 – 3.49 (m, 1120H), 2.43(s, 6H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 144.8, 133.5, 129.9, 128.1, 70.6, 69.3, 68.7, 21.6.

### PEG (12k) -(N<sub>3</sub>)<sub>2</sub> (45)

This was obtained according to general procedure B. **Compound 44** (7.2g, 0.585 mmol), 50 mL of distilled H<sub>2</sub>O: THF (80:20 %v/v) and NaN<sub>3</sub> (114 mg, 1.75mmol) were used in the reaction. As a final point, the product was purified by silica gel column chromatography using (5:1 v/v) CH<sub>2</sub>Cl<sub>2</sub>: MeOH as an eluent to obtain a white powder with 81.2 % of yield. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 3.73 – 3.44 (m, 1120H), 3.34 (t, *J* = 5.1 Hz, 4H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 70.5, 70.0, 50.6.

### PEG (12k) -(NH<sub>2</sub>)<sub>2</sub> (46)

This was obtained according to general procedure C. **Compound 45** (100 mg, 0.0083mmol), 15 mL of MeOH, Pd/C (20 mg) were used. The reaction was stirred for 5 days to obtain a product with 96.4%. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 3.74 – 3.58 (m, 1120H), 3.45 (t, *J* = 5.2 Hz, 4H), 2.87 (t, *J* = 5.2 Hz, 4H), 1.78(s, 4H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 73.6, 70.6, 69.9, 41.9

### PEG (12k) -(maleimide)<sub>2</sub> (47)

This is synthesized according to general procedure D. **Compound 1** (3.2 mg, 0.0174mmol), NMM (2.13 mg, 0.0182mmol), <sup>i</sup>BuCoCl (2.48mg, 0.0182mmol.) and **Compound 46** (95 mg, 0.0079mmol) and 15 mL CH<sub>2</sub>Cl<sub>2</sub> were used to obtain a white solid product with 57.5% of yield. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 6.70 (s, 4H), 6.32 (s, 2H), 3.79 –

3.61 (m, 1120H), 3.62-3.53 (m, 8H), 3.47 (t,  $J = 5.2$  Hz, 4H), 2.33 (t,  $J = 7.2$  Hz, 4H), 1.99 – 1.87 (m, 4H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  172.0, 170.9, 134.2, 70.7, 70.3, 70.0, 39.3, 37.2, 33.4, 24.9.

#### PEG (12k)-[dG<sub>2</sub>]<sub>2</sub> (48)

This was synthesized according to general procedure E. G<sub>2</sub> (29.9mg, 0.0089mmol), 5 mL of PBS (1x, pH 6.5), TCEP (25.5mg, 0.089mmol), **Compound 47** (100 mg, 0.0081 mmol) dissolved in 5 mL of H<sub>2</sub>O and few drops of DMSO. The purification was performed gel filtration chromatography using PD MidiTrap G-25 columns. The product was isolated as white powder with 51.2 % yield.  $^1\text{H}$  NMR (400 MHz, D<sub>2</sub>O)  $\delta$  3.81 – 3.61 (m, 1120H), 3.66– 3.60 (m, 8H), 3.58– 3.25 (m, 88H), 3.17 (t,  $J = 6.0$  Hz, 8H), 2.91 – 2.82 (m, 56H), 2.80 – 2.69 (m, 30H), 2.61 – 2.46 (m, 56H), 2.23– 2.14 (m, 8H), 1.92 – 1.81 (m, 4H).  $^{13}\text{C}$  NMR (101 MHz, D<sub>2</sub>O)  $\delta$  179.17, 178.95, 172.84, 172.84, 172.42, 77.48, 77.16, 77.16, 76.84, 69.8, 69.8, 51.9, 49.7, 49.5, 39.1, 38.9, 37.0, 34.4, 30.4, 28.9, 27.7, 22.8, 22.1.

#### PEG (12k)-[dG<sub>2</sub>-(AXO)<sub>8</sub>]<sub>2</sub> (49) : **BiAn(12000)**

This was obtained by following general procedure F. **Compound 48** (64 mg, 0.0041mmol) and AX (50.6 mg, 0.131 mmol) were reacted. The reaction was refreshed with 10 mg of AX on three different occasions. After purification the product was collected as yellowish powder with 53.9 % of yield.  $^1\text{H}$  NMR (400 MHz, D<sub>2</sub>O)  $\delta$  7.29 (d,  $J = 7.8$  Hz, 32H), 6.89 (d,  $J = 7.8$  Hz, 32H), 4.94 (d,  $J = 8.0$  Hz, 16H), 4.63 (s, 16H), 4.29 (d,  $J = 8.0$  Hz, 16H), 3.88 – 3.57 (m, 1120H), 3.56 – 3.51 (m, 8H), 3.34 (s, 16H), 3.32 – 3.05 (m, 88H), 3.12 – 3.03 (m, 8H), 2.89 – 2.69 (m, 56H), 2.66 – 2.51 (m, 30H), 2.48 – 2.26 (m, 56H), 2.18 – 2.06 (m, 8H), 1.90– 1.77 (m, 4H), 1.46 (s, 48H), 1.19 (s, 48H).  $^{13}\text{C}$  NMR (101 MHz, D<sub>2</sub>O)  $\delta$  175.6, 175.1, 174.6, 171.6, 156.2, 129.7, 128.8, 115.9, 77.1, 74.9, 69.8, 69.6, 68.9, 64.9, 59.5, 59.1, 58.2, 39.4, 38.9, 38.7, 36.9, 36.9, 32.7, 28.9, 28.2, 26.8, 23.6, 23.0.

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