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Generation and Characterization of Anti-Glucosepane Antibodies Enabling Direct Detection of Glucosepane in Retinal Tissue

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Abstract

Although there is ample evidence that the advanced glycation end-product (AGE) glucosepane contributes to age-related morbidities and diabetic complications, the impact of glucosepane modifications on proteins has not been extensively explored due to the lack of sufficient analytical tools. Here, we report the development of the first polyclonal anti-glucosepane antibodies using a synthetic immunogen that contains the core bicyclic ring structure of glucosepane. We investigate the recognition properties of these antibodies through ELISAs involving an array of synthetic AGE derivatives and determine them to be both high-affinity and selective in binding glucosepane. We then employ these antibodies to image glucosepane in aging mouse

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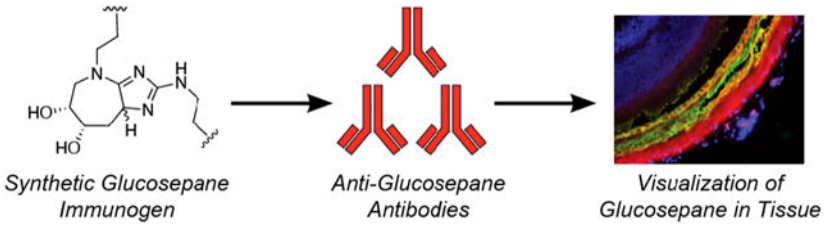
retinae via immunohistochemistry. Our studies demonstrate for the first time accumulation of glucosepane within the retinal pigment epithelium, Bruch's membrane, and choroid: all regions of the eye impacted by age-related macular degeneration. Co-localization studies further suggest that glucosepane colocalizes with lipofuscin, which has previously been associated with lysosomal dysfunction and has been implicated in the development of age-related macular degeneration, among other diseases. We believe that the anti-glucosepane antibodies described in this study will prove highly useful for examining the role of glycation in human health and disease.

Graphical Abstract



INTRODUCTION

Advanced glycation end-products (AGEs) are a heterogeneous group of compounds formed as a result of non-enzymatic reactions between nucleophilic residues and sugars. AGEs have been shown to alter the structure of long-lived proteins, such as crystallin, albumin, and collagen,[1] making them more resistant to degradation and promoting their accumulation in cells and tissues with age.[2–5] There is increasing evidence that AGEs are involved in the pathogenesis of age-related and chronic diseases, including age-related macular degeneration (AMD) and diabetes.[6–9]

Glucosepane is among the most abundant AGEs found in human tissues. It is formed from lysine, arginine, and glucose, and it is over an order of magnitude more abundant than any other AGE cross-link in the extracellular matrix (ECM; Supplementary Scheme 1).[10] Notably, glucosepane levels have been shown to correlate with various disease states, including diabetic retinopathy, microalbuminuria, and neuropathy.[3,11–13] While the exact mechanisms behind glucosepane-mediated dysfunction remain unclear, it is believed to impair the functional and mechanical properties of proteins in the ECM[14] and interfere with proteolytic degradation of collagen.[15]

To date, the primary method for identifying glucosepane in tissues has required exhaustive enzymatic degradation followed by high pressure liquid chromatography−mass spectrometry (LC/MS).[13,16,17] Although these protocols have proven effective in quantifying glucosepane in bulk tissue extracts, they are labor-intensive, and the degradation process destroys the tissue architecture, making it difficult to examine the localization of glucosepane.

In recent years, anti-AGE antibodies have emerged as useful tools for studying AGEs and have the advantage of being compatible with the evaluation of intact tissues, enabling immunohistochemical staining and imaging procedures.[18] nb Several anti-AGE antibodies have been produced by immunization of animals with AGEs generated either from total

synthesis[19] or through *in vitro* glycation methods. Such methods involve the incubation of an immunogenic carrier protein, such as BSA, with glucose or other reactive sugar metabolites.[20–23] Reaction conditions that generate glucosepane[24] are known also to generate a range of AGE byproducts, including carboxymethyllysine.[25,26]

These *in vitro* preparation methods are unlikely to produce antibodies that are specific for glucosepane, although no such studies have been reported. To avoid this expected complication, we decided to synthesize homogeneous, synthetic glucosepane immunogens.

Herein, we describe the development and characterization of the first antibodies known to selectively recognize glucosepane. To this end, we have created a synthetic glucosepane immunogen that closely resembles glucosepane found *in vivo* and used it to generate a polyclonal antibody serum that recognizes glucosepane both *in vitro* and in *ex vivo* tissue samples. We have demonstrated that the antibodies can bind to glucosepane with high degrees of specificity and sensitivity through ELISA studies and have employed these antibodies in immunohistochemical experiments. Interestingly, these latter studies demonstrate that glucosepane accumulates within subcomponents of the retina, specifically the retinal pigment epithelium (RPE), Bruch's membrane, and choroid, which are anatomic areas highly affected by AMD and diabetic retinopathy.[27,28] Our results suggest that anti-glucosepane antibodies could be useful for uncovering mechanisms through which glucosepane contributes to aging and disease and could potentially serve as tools for the diagnosis of aging and diabetic complications.

RESULTS AND DISCUSSION

Immunogen Design and Synthesis.

In order to generate antibodies capable of binding glucosepane cross-links in proteins, we designed immunogen **1** (Scheme 1a), containing the glucosepane core incorporated into an immunogen peptide as our target epitope (highlighted in red, Scheme 1a). Surrounding the target epitope, we included polyethylene glycol and glycine residues. We hypothesized that these motifs would only contribute minimally to antibody binding affinity due to their flexibility and, as such, would allow us to obtain clones that interact preferentially with the target epitope. Cysteine was added to the C-terminus as a chemical handle to enable thiol-maleimide conjugation reactions to proteins and solid supports.

Immunogen **1** (Scheme 1a) was retrosynthetically disconnected in a manner to facilitate late-stage incorporation of advanced precursor **2** (Scheme 1a) into the peptide sequence. The key disconnection was made between the 2-amino group and the rest of the bicyclic glucosepane core. We hypothesized that we could construct this bond through a late-stage reaction of an unprotected ornithine side-chain with the electrophilic glucosepane precursor **2** using a strategy derived from our reported glucosepane total synthesis (Supplementary Figure 1).[29] This, in turn, could be appended to resin-bound ornithine derivative **3**, which could be readily prepared via solid-phase peptide synthesis (SPPS). We felt this late-stage installation of glucosepane precursor **2** would be more material-efficient than a traditional monomer-based approach with an Fmocprotected glucosepane amino acid monomer (Supplementary Figure 1).

For the synthesis of electrophilic glucosepane precursor **2**, we followed a synthetic strategy similar to what we employed previously in the total synthesis of glucosepane (Supplementary Scheme 2).[29] This 10-step procedure starts from readily available 1,5-dibromopentane and affords antigen precursor **2** with an overall yield of 3.6%. Compound **2** was then allowed to react with resin-bound ornithine derivative **3** to afford a 2,4-diamino-5-hydroxyimidazole species on-resin. After a sodium triacetoxyborohydride reduction and cleavage from the resin, we accessed the desired glucosepane immunogen **1** in 4.06% yield.

Antibody Generation and Characterization.

Upon completion of synthesis, peptide immunogen **1** was conjugated to keyhole limpet hemocyanin (KLH) *via* the pendant thiol (Scheme 1b). This conjugate was then used by New England Peptide, Inc. (Gardner, MA) to immunize New Zealand white rabbits. After an immunization period of three months, serum samples were collected from the rabbits. The polyclonal antiserum was then subjected to negative selection against oxidized glucosepane derivative **4** to remove antibodies specific for the peptide backbone, followed by positive selection against agarose beads coated with glucosepane immunogen **1.** Following elution, we isolated anti-glucosepane antibodies.

The specificity of the purified anti-glucosepane antibodies was tested *via* ELISA against a panel of synthetic peptides (Figure 1a, **6−9)** containing some of the most abundant AGEs, along with arginine-containing (Figure 1a, **5**) and glucosepane-containing controls (Figure 1a, **10**).[30,31] Peptides were anchored to the wells of maleimide-coated 96-well plates *via* thiol-maleimide conjugation reactions. Binding of these synthetic peptides was then tested against the polyclonal serum. As hypothesized, peptides **5−9** exhibited no detectable binding to the antibodies, while the glucosepane peptide **10** bound tightly to the antibodies with an EC_{50} of 14.16 ± 0.20 nM (Figure 1b). To the best of our knowledge, this is the first report of the generation of glucosepane-specific antibodies.

To characterize the binding epitope of the antibodies further, additional ELISAs were performed with several abiotic glucosepane analogs. These experiments were designed to determine the importance of various structural elements of glucosepane toward the binding epitope of the antibodies. As expected, the glucosepane peptide most structurally similar to the immunogen (**S24**) exhibited the lowest EC_{50} for the antibodies at 5.6 ± 0.23 nM. Scaffolds that slightly perturbed the core immunogen scaffold demonstrated weaker affinities. For example, when the hydroxyl groups were not present (as in **S22**), the EC_{50} increased to 7.07 ± 0.13 nM, and when the core ring system was oxidized (as in **S23**), the EC_{50} increased to 11.86 ± 0.20 nM. Further differences, such as the absence of the polyethylene glycol motif in addition to the hydroxyl groups (as in **S21**), resulted in a more significant reduction in affinity to ~1 μM. Additional alterations, such as cleavage of the seven-membered ring (as in **S20**) caused the EC_{50} of the antibodies to rise to levels comparable to negative control compound **5** (Supplementary Figure 2).

In summary, the only analogs that showed significant affinity to the antibodies are abiotic. Two substrates in physiological systems that have the potential to cross-react with the antibodies generated herein are pentosinane and pentosidine, but to date there have been no

reported synthetic routes to peptides containing these modifications.[32] The abiotic analogs tested also offer insights into our antibody generation and purification process. The high binding affinity of oxidized substrate **S23** highlights that negative affinity purification did not completely precipitate antibodies capable of binding to this substrate. Meanwhile, PEGylated **S24** highlights that PEGylation of the immunogen results in a minor contribution to the binding epitope over AcHN-Lys-NHMe-modified **10** (5.6 ± 0.23 nM, **S24;** 14.16 ± 0.20 nM, **10**).

An experiment was also performed to demonstrate the capacity of a soluble inhibitor **S25** to inhibit binding of the antibodies to glucosepane substrate **S24.** When excess soluble glucosepane competitor **S25** was present, the binding of the antibodies was reduced to background levels (Supplementary Figure 3).

Overall, these experiments demonstrate that antibody binding is highly specific for the molecular structure of glucosepane, and comparable binding properties are observed only for very closely related abiotic analogs.

Having demonstrated that the antibodies bound synthetic glucosepane specifically and with high affinity, we next sought to evaluate their utility in biological samples. To this end, we measured glucosepane levels in several commercial samples of human serum albumin (HSA) using an LC/MS method. Approximately 0.3% of the arginine residues in these HSA samples were found to be modified by glucosepane. We also employed *in vitro* glycation methods to enrich glucosepane content in HSA and found these samples to contain glucosepane levels of 1.3%.[33]

ELISAs were then performed, wherein our antibodies were exposed directly to low- and high-content HSA samples. As predicted, significantly greater binding was observed with glycated HSA compared to commercially available HSA (Figure 1c). Importantly, the glucosepane levels used in this experiment, 0.3% and 1.3%, align with the range of glucosepane levels that have been observed in human serum samples.[16]

Glucosepane in Aging Retinae.

Glucosepane has been found to accumulate in various tissues with age[10,16] and has long been thought to play a role in promoting ocular diseases such as cataracts, diabetic retinopathy, and AMD.[3,12,34] However, tissue localization studies of glucosepane have not been performed to our knowledge. Therefore, we next sought to apply the anti-glucosepane antibodies to study the localization of glucosepane in the retinae of aging mice *via* immunohistochemistry. In these studies, we sectioned, stained, and imaged retinae of young (2 month), middle-aged (7 month), and older (12 month) C57BL/6J mice (Figure 2a). These experiments revealed an increase in the area of glucosepane staining in retinal tissue samples with age. Further examination of the IHC images showed glucosepane accumulation in the retinal pigment epithelium (RPE), Bruch's membrane, and choroid: regions associated with diseases such as AMD and retinopathy (Figure 2b).[27,28] Weaker glucosepane staining was also observed in the photoreceptor inner and outer segments (Figure 2a). Additionally, in competition experiments, a reduced fluorescence signal was observed in the presence

of peptidic competitor **S25** (Supplementary Figure 3), suggesting that the antibodies were binding glucosepane cross-links.

Notably, granular glucosepane staining was observed in the RPE at the subcellular level (Figure 2b). We, therefore, hypothesized that there could be colocalization between glucosepane and lipofuscin, a lysosomally derived, non-degradable, heterogeneous molecular aggregate that is partially characterized by its granular appearance. Previous reports have proposed that the accumulation of lipofuscin within the RPE is considered to be a risk factor for age-related diseases of the eye.[35] Lipofuscin has been shown to induce lysosomal dysfunction and interfere with autophagic clearance, contributing to the development of various eye pathologies, including AMD.[36–39] There is also evidence that AGEs promote formation of lipofuscin and that AGE-modified proteins may be components of lipofuscin granules.[40,41]

Lipofuscin granules are often identified by their characteristic autofluorescence, as well as their lysosomal origin, indicated by colocalization with lysosomal-associated membrane protein 1 (LAMP-1).[42] After partial bleaching of retinal samples, colocalization between autofluorescent granules and glucosepane was observed in regions of the RPE (Figure 3a). Additional costaining experiments demonstrated colocalization between glucosepane and LAMP-1 (Figure 3b). Although other AGEs have been associated with lipofuscin formation,[43,44] this is the first evidence for the colocalization of lipofuscin and glucosepane. Our findings therefore suggest that glucosepane-modified proteins may be constituents of lipofuscin and accumulate within the RPE with age. Given the known involvement of lipofuscin in disease, these findings warrant further investigations into the impact of glucosepane accumulation on lysosomal activity and disease onset. These data also suggest that glucosepane merits exploration as a biomarker for age-related eye disease.

CONCLUSIONS

Investigation into the role of AGEs in pathophysiology has been hindered by a lack of tools to study the distribution of individual AGEs in intact tissues. Through the preparation of a synthetic glucosepane immunogen, we have developed the first polyclonal anti-glucosepane antibodies, which recognize glucosepane with high specificity and sensitivity. These antibodies bind both synthetic glucosepane and glucosepane-like molecules, as well as glucosepane-modified HSA in a manner that correlates with traditional HPLC/MS protocols. Furthermore, we applied these antibodies to demonstrate, for the first time, that glucosepane accumulates in the RPE, Bruch’s membrane, and choroid in the retinae of aging mice. The accumulation of glucosepane in the RPE partially colocalizes with lipofuscin granules, suggesting a potential link to lipofuscin-related pathology. Taken together, these data demonstrate that anti-glucosepane antibodies are effective tools for examining glucosepane cross-links in tissue with subcellular resolution. In the future, these antibodies will serve as tools to provide insight into the role of glucosepane in human health and disease.

METHODS

A description of all chemicals, reagents, instrumentation, and procedures is available in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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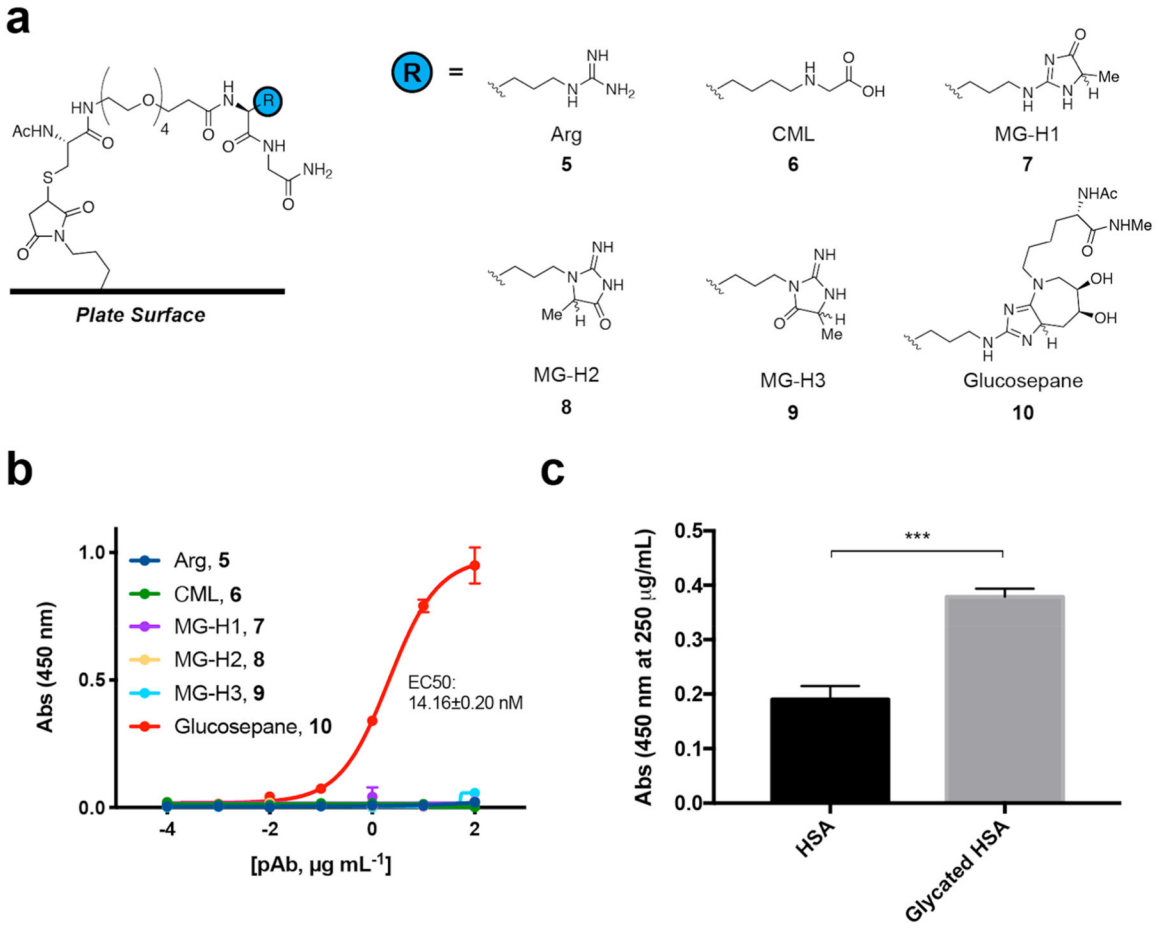


Figure 1.

Chemical structures of synthetic ELISA substrates and the corresponding ELISA results used to demonstrate anti-glucosepane antibody selectivity. (a) Synthetic peptides containing arginine, other advanced glycation end-products, and glucosepane (CML = carboxymethyllysine; MG-H1 = methylglyoxal-derived hydroimidazolone 1; MG-H2 = methylglyoxal-derived hydroimidazolone 2; MG-H3 = methylglyoxal-derived hydroimidazolone 3). (b) ELISA data toward panel of synthetic peptides. Indirect ELISAs were run on maleimide-coated plates. Error bars represent standard deviation ($n = 2$). (c) Protein-based indirect ELISA on Nunc plates. Binding of anti-glucosepane antibodies was tested against commercially available HSA and glycated HSA (***$P < 0.0005$, two-tailed, unpaired *t* test).

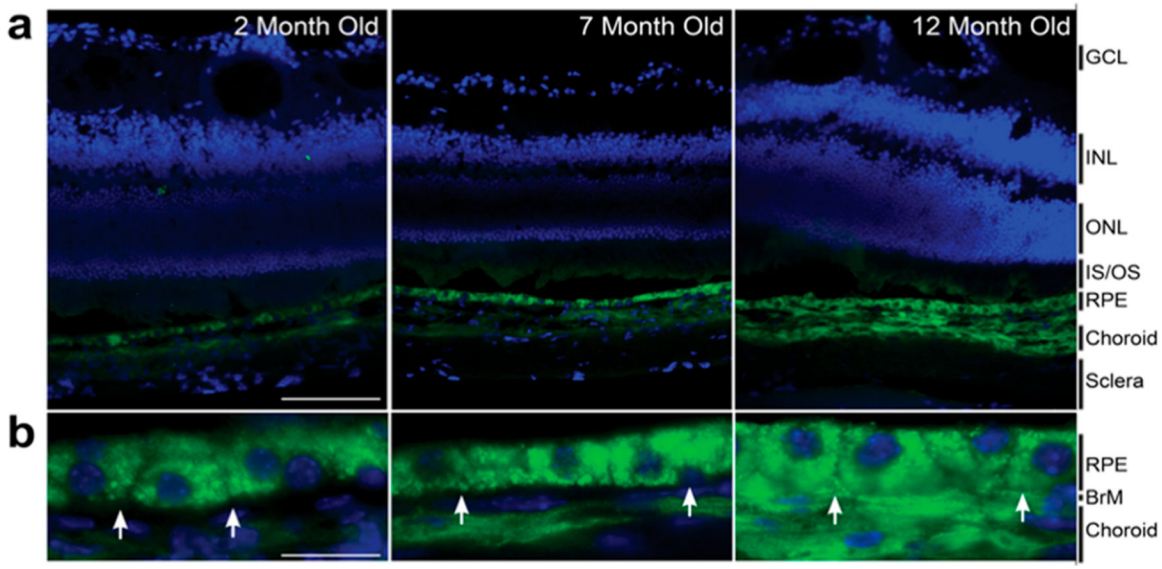


Figure 2.

Immunohistochemical images of mouse retinae. Retinal samples were taken from 2, 7, and 12 month old mice. (a) Representative retinal sections labeled with anti-glucosepane antibody/anti-Rbt Alexa488 (green) and DAPI (blue). Scale bars are 100 μm (GCL = ganglion cell layer; INL = inner nuclear layer; ONL = outer nuclear layer; IS/OS = inner/outer segments; RPE = retinal pigmented epithelium). (b) Representative high-magnification images of the RPE, Bruch's membrane, and choroid. White arrows highlight the position of Bruch's membrane (BrM). Scale bars are 20 μm.

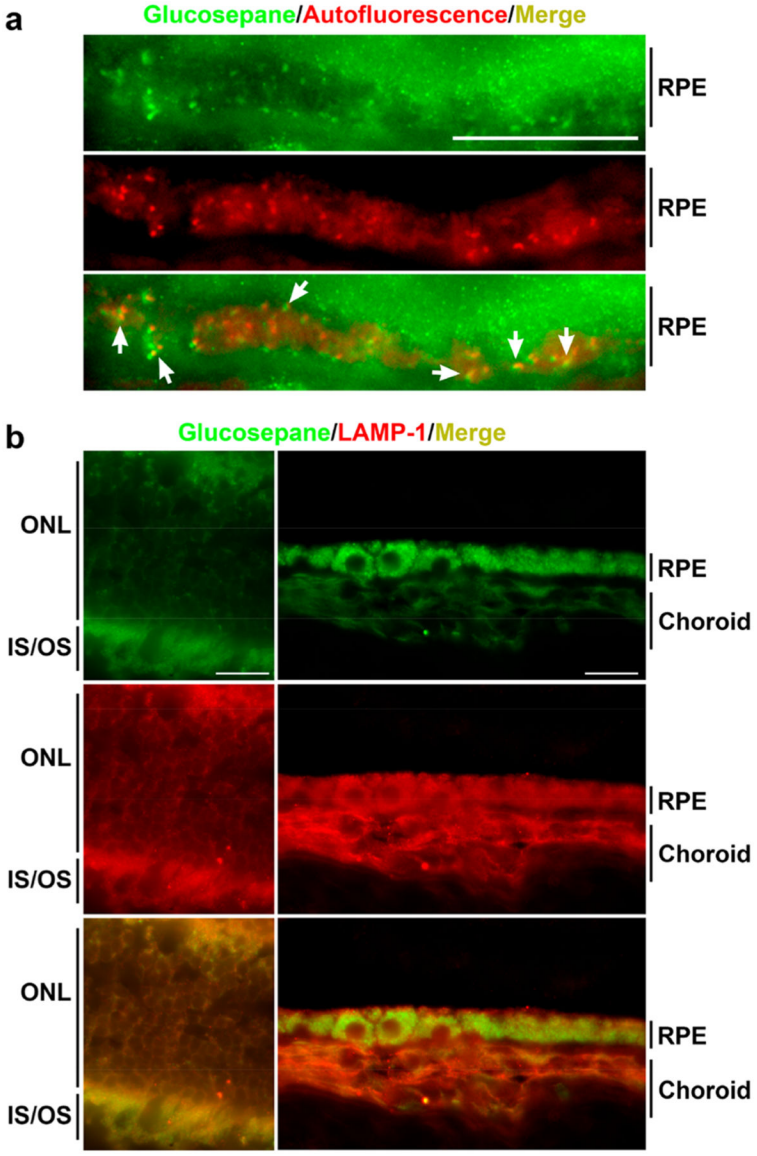
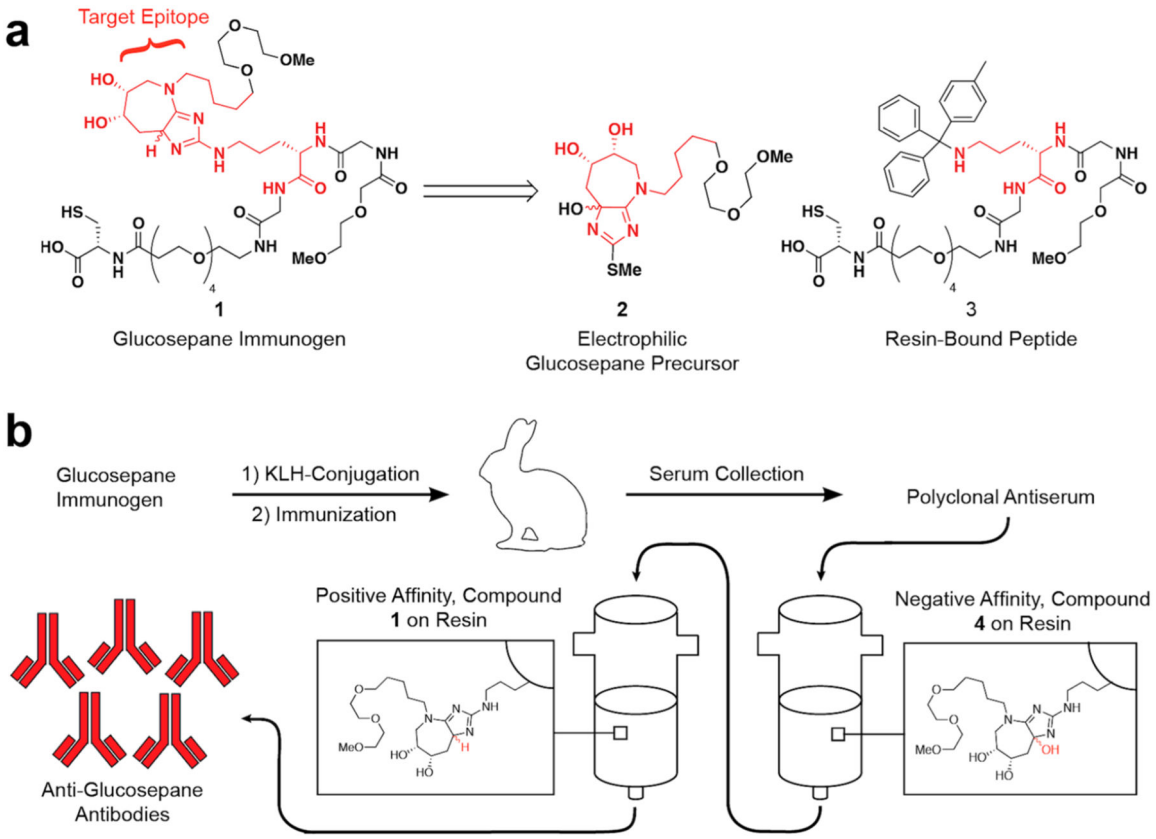


Figure 3.

Immunohistochemical images of 12-month-old mouse retinae. (a) Localization of glucosepane (green) and autofluorescence (red) in partially bleached RPE. Yellow indicates colocalization. White arrows highlight areas of colocalization. (b) Localization of glucosepane (green) and LAMP1 (red) in unbleached retinal photoreceptors (left) and bleached RPE/choroid (right). Yellow indicates colocalization. Scale bars are 20 μm (ONL = outer nuclear layer; IS/OS = inner/outer segments; RPE = retinal pigmented epithelium).



Scheme 1.

(a) Immunogen Retrosynthesis[a] and (b) Antibody Generation and Purification Process[b]

[a]Immunogen **1** was accessed from electrophilic glucosepane precursor **2** and resin-bound peptide **3.** [b]The immunogen was conjugated to keyhole limpet hemocyanin (KLH). Rabbits were immunized, and antiserum was collected. The antiserum was subjected to a series of affinity purifications, and the resulting antibodies were used for all subsequent experiments.