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Note

Variability in the carbazole assay for N-desulfonated/N-acylated heparin derivatives

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Abstract—The carbazole assay is commonly employed to quantify heparin and other uronic acid-containing polysaccharides. Heparin-derived standard curves are often employed to quantify solutions of various natural and unnatural heparin structures that have different levels of sulfate substitution, different levels of *N*-sulfo and *N*-acetyl groups, and other structural changes as a consequence of reducing molecular weight. Recent studies in our laboratory have focused on chemically modified heparin derivatives comprised of structurally diverse *N*-acyl moieties substituted into heparin in place of *N*-sulfo groups. We report here that although differing degrees of 2-*N*-sulfo-, 2-*N*-acetyl- or 2-amino-D-glucosamine residues within heparin do not affect signal intensity in the carbazole assay, replacing *N*-sulfo groups in heparin with structurally diverse *N*-acyl moieties affords products that display significant variation in the assay. The structure of different *N*-acyl groups, and to a lesser extent the degree of N-acylation by individual *N*-acyl groups, is shown to variably alter signal intensity in the carbazole assay even though content and structure of uronic acid residues is unaltered.

Keywords: Carbazole assay; Heparin; Heparin derivatives

The uronic acid carbazole reaction was originally developed over 50 years ago by Dische as a specific and sensitive colorimetric test for uronic acids.[1–4] The carbazole assay as a method for quantifying uronic acid residues in oligosaccharides and polysaccharides involves heating the saccharide in concentrated sulfuric acid to cleave glycosidic bonds, followed by heating with carbazole to yield a violet color, $\lambda_{max} = 530$ nm. It is well understood that heating polysaccharides in concentrated sulfuric acid gives rise to furan compounds. In the case of polyuronides, furoic acids form from the heating of the polymer in acid and interact with carbazole.[5] The addition of borate and adaptation to 96-well plates makes the assay more sensitive and useful as an inexpensive method for determining solution concentrations of certain saccharides.[6,7]

To use the carbazole assay as a quantitative tool, a standard (usually glucuronic acid, glucuronolactone, or corresponding polyuronide/polysaccharide) is employed.[1,2,6,8] In theory, a uronic acid standard of known concentration should be proportional to a certain polyuronide sample of unknown uronide content because the glycosidic bonds in the polysaccharide are cleaved. However, it is known that differences in glycosidic linkages and the presence of different monosaccharide residues can alter color yield.[1,2,4,6–8] The carbazole assay is regularly employed to quantify solution concentrations of heparin and chemically modified heparins.[1,6,9,10] Heparin is composed of repeating β-D-glucopyranosiduronic acid or α-L-iodopyranosiduronic acid residues (1→4) linked to *N*-acetyl or *N*-sulfo α-D-glucosamine residues, and is variably substituted with *O*-sulfo (sulfate) groups (Fig. 1). The N-desulfonation of heparin has been shown to afford no change in color intensity compared to heparin, while heparin amides expectedly exhibit low color yields.[9,10]

We recently demonstrated that the *N*-sulfo groups on heparin can be replaced with structurally diverse non-anionic moieties to yield charge-reduced heparin

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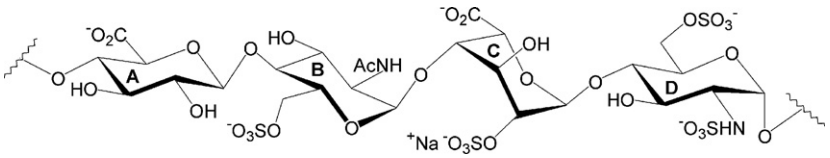


Figure 1. Representative tetrasaccharide within heparin polysaccharide depicting the most prevalent residues. (**A**) β-D-glucuronic acid, (**B**) *N*-acetyl-α-D-glucosamine, (**C**) α-L-iduronic acid, (**D**) *N*-sulfo-α-D-glucosamine.

derivatives that bind to certain heparin-binding proteins without loss of affinity.[11,12] During analyses to characterize these structurally diverse N-desulfonated/N-acylated heparin derivatives, and efforts to quantify solution concentrations of these novel heparins, it was observed that different *N*-acyl moieties substituted into heparin in place of *N*-sulfo groups afforded unpredictable intensity changes in the carbazole assay. This observation suggested that quantifying solution concentrations of each novel heparin derivative using the carbazole assay might require individual standard curves for each *N*-acyl heparin, unlike heparin, many natural variants of heparin, and low molecular weight heparins, which are regularly quantified in solution using heparin-based standard curves.

In this work we set out to answer the following questions regarding application of the carbazole assay toward quantifying chemically modified heparin derivatives bearing different *N*-acyl structures in place of *N*-sulfo groups: (1) What is the degree of variation in the assay between different *N*-acyl structures substituted into heparin in place of *N*-sulfo groups? (2) What is the degree of variation in the assay for individual *N*-acyl groups substituted into heparin in place of varied percentages of *N*-sulfo groups? By answering these questions we looked to determine if quantifying different *N*-acyl heparin derivatives by the carbazole assay required each derivative to be viewed as a unique polysaccharide, or if trends would emerge to allow potential standardization of the carbazole assay across different N-acylated heparins.

We first established that varying the percent of *N*-sulfo groups versus amine groups in heparin does not contribute to variations in color yield because initial observations suggested percent N-desulfonation/N-acylation affected signal intensity in the assay and in baseline controls. Heparin derivatives with varying percentages of N-desulfonation, percent free amine (**N12** (12%), **N50** (50%), and **N100** (100%)), were compared with and without carbazole at two different concentrations, 25 and 37.5 μg/well (Fig. 2). Percent N-desulfonation of heparin (% free amine) has no significant effect on absorbance intensity in the assay (Fig. 2A and B), or in controls having no carbazole (Fig. 2C and D). These results confirm that varied degrees of N-desulfonation of heparin, without subsequent N-acylation, affords products that are indistinguishable from parent heparin in the carbazole assay.[10]

Ten different 100% N-desulfonated/N-acylated heparin derivatives (**D1**–**D10**, Table 1) were evaluated at equivalent concentrations in the carbazole assay to investigate the effect of different *N*-acyl moieties on color yield. Color yields for derivatives **D1**–**D10** reveal that different *N*-acyl groups afford variation in color yield (Fig. 3A). Derivatives **D4** and **D10** display significantly lower color yield in comparison to heparin and N-desulfonated/N-acetylated heparin (**D1**). Other *N*-acyl groups display smaller variations in color yield that are not significantly different than heparin.

Each of the ten *N*-acyl heparin derivatives was subjected to assay conditions without carbazole to determine if individual *N*-acyl groups on heparin inherently afford products that absorb at 530 nm or otherwise might contribute to, or interfere with, assay signal. Indeed a significant difference in this baseline color yield for select N-acylated heparins is observed when carbazole-free ethanol is used is place of ethanol containing 0.125% carbazole (Fig. 3B). This result demonstrates that certain *N*-acyl groups substituted into heparin in place of *N*-sulfo groups inherently affords products that break-down or otherwise react under assay conditions to afford dramatic effects on absorbance at 530 nm. The contribution of increased baseline signal approaches 10% of total signal (**D7** and **D10**, Figure 3). No direct correlation of the baseline signal to increased or decreased assay signal is observed.

Because certain *N*-acyl groups substituted into heparin in place of *N*-sulfo groups displayed significant differences in signal intensity in the carbazole assay, it was anticipated that the degree of N-desulfonation/N-acylation with these *N*-acyl groups should also afford a corresponding increase or decrease in color yield. To test this, derivatives of two different *N*-acyl heparins (**D1** and **D10**) having varied degrees of N-desulfonation/N-acylation were evaluated in the carbazole assay (Fig. 4). No significant effect on signal intensity was observed for derivatives having varied percentages of *N*-sulfo groups replaced with *N*-acetyl moieties (**D1**, Figure 4A), demonstrating that signal intensities for N-desulfonated/N-acetylated heparin are consistent with heparin and N-desulfonated heparin. In contrast to *N*-acetyl substitution, increasing the percentage of *N*-sulfo groups substituted with the arylacyl moiety in

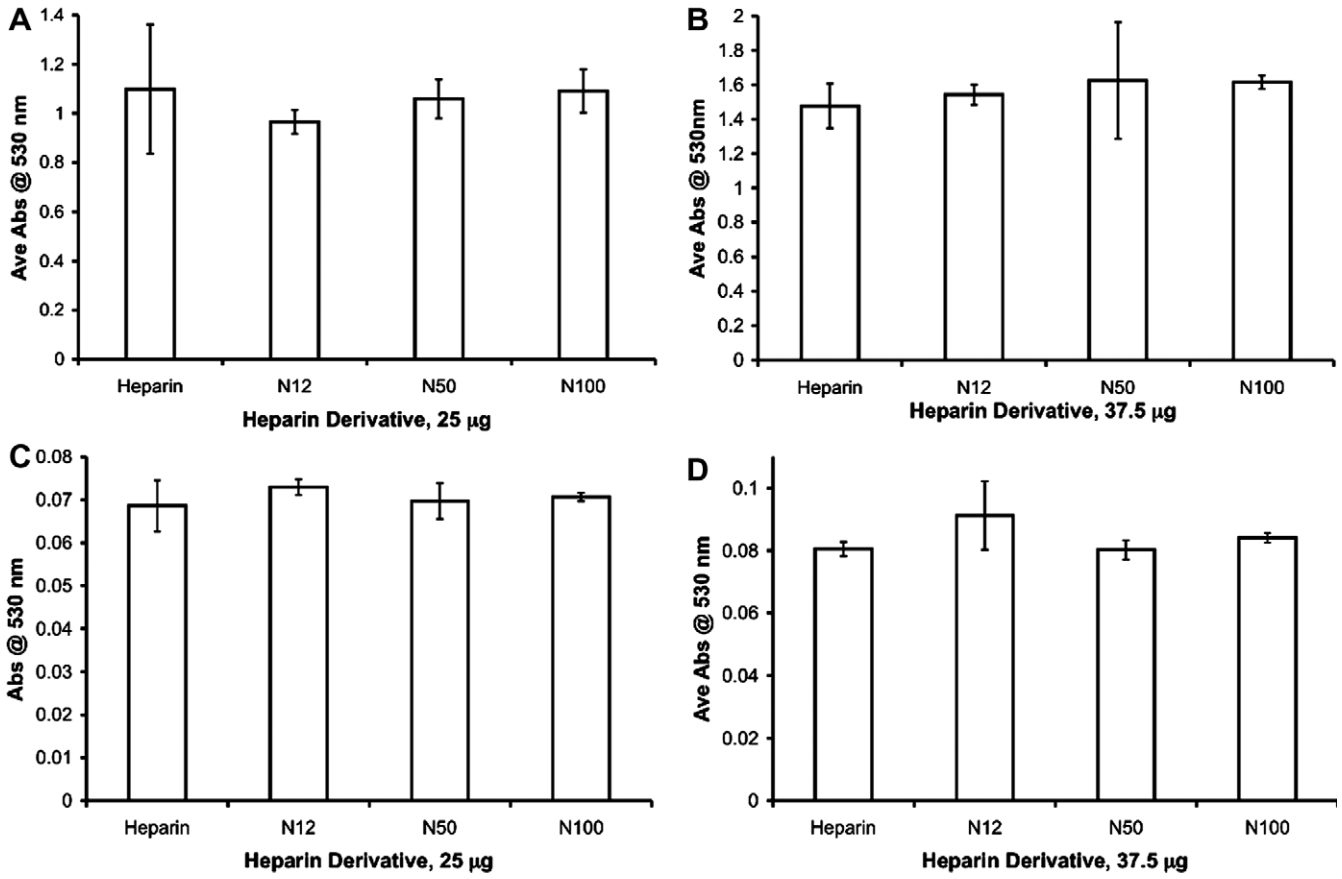
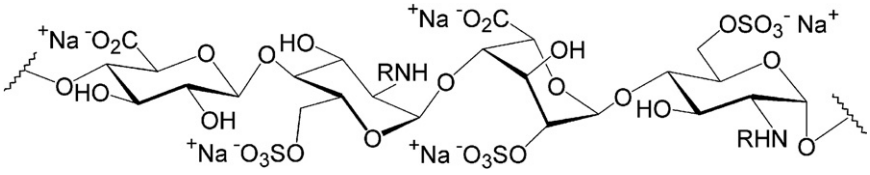
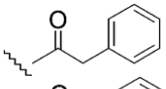
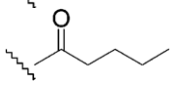
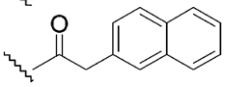
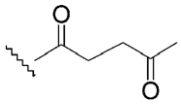
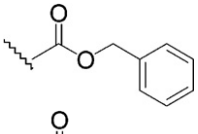
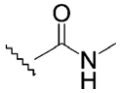
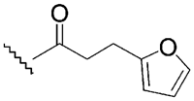
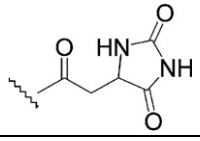
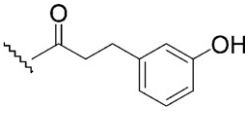


Figure 2. Carbazole assay analysis of heparin and three heparin derivatives of varying percent N-desulfonation (percent free amine): **N12** (12%), **N50** (50%), and **N100** (100%). (A) 25 μg/well in the presence of carbazole. (B) 37.5 μg/well in the presence of carbazole. (C) 25 μg/well without carbazole. (D) 37.5 μg/well without carbazole.

Table 1. Structures of N-desulfonated/N-acylated heparin derivatives evaluated



R=	Heparin derivative	R=	Heparin derivative
	**D1**		**D6**
	**D2**		**D7**
	**D3**		**D8**
	**D4**		**D9**
	**D5**		**D10**

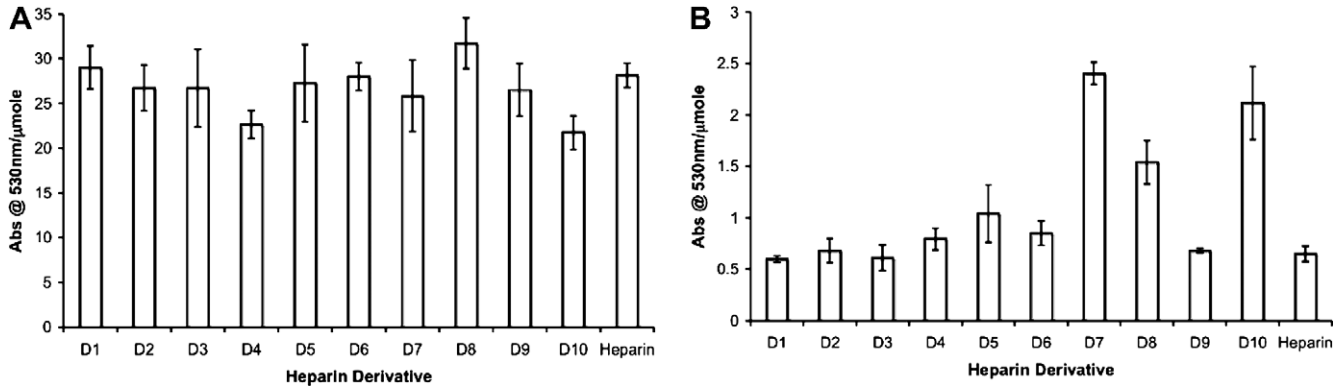


Figure 3. Comparison of color yields from carbazole assay analysis of ten different 100% N-desulfonated/N-acylated heparin derivatives (**D1**–**D10**) and heparin in the presence (A) and absence (B) of carbazole.

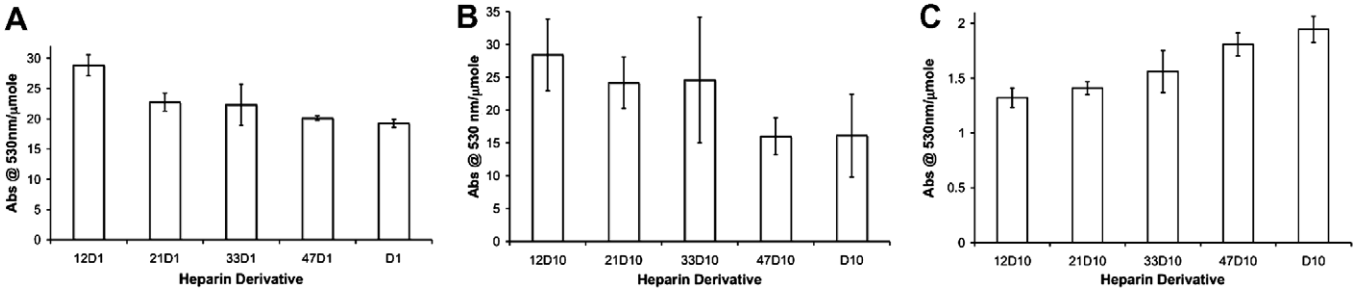


Figure 4. Affect of percent N-desulfonation/N-acylation of heparin on color yields in the carbazole assay. (A) Replacing 12–100% of the *N*-sulfo groups in heparin with *N*-acetyl groups (**D1**). (B) Replacing 12–100% of the *N*-sulfo groups in heparin with arylalkyl *N*-acyl group (**D10**). (C) Baseline control data for **D10** derivatives in panel B employing carbazole-free ethanol.

D10 revealed a trend toward decreasing signal intensity with increasing level of N-acylation (Fig. 4B), which is consistent with **D10** affording the lowest color yield of the ten 100% N-desulfonated/N-acylated heparin derivatives (Fig. 3A). Increasing the percentage of *N*-sulfo groups substituted with **D10** revealed a consistent increase in signal intensity in the absence of carbazole (Fig. 4C), which is consistent with the higher absorbance values observed in the corresponding single-concentration studies (Fig. 3B).

Having demonstrated N-desulfonation/N-acylation of heparin with some, but not all, *N*-acyl groups alters signal intensity in the carbazole assay, we compared linearity and slope of standard curves for **D1** and **D10** with heparin. The 100% N-desulfonated/N-acetylated heparin derivative **D1**, which afforded signal intensities equivalent to parent heparin and N-desulfonated heparins, shows a linear relationship and slope comparable to heparin over the concentration range of 2.5–50 μg/well (Fig. 5). In contrast, slope of the standard curve for 100% N-desulfonated/N-acylated heparin derivative **D10** is significantly decreased (Fig. 5).

Despite historical utility of the carbazole assay to quantify solution concentrations of heparin and heparin derivatives using heparin-derived or other urinate-derived standard curves, the studies here demonstrate that chemical modification of heparin through N-desulfonation/N-acylation affords products with unpredictable variations in color yield in this assay, even when these modifications do not alter content or structure of the uronic acid residues. Differences in signal intensity have been found to vary with structure of the *N*-acyl moiety in the absence of any other differences in saccharide structure. It is also demonstrated that signal intensity can vary as a consequence of degree of N-acylation.

An increasing number of reports describe chemically modified heparin and other chemically modified polysaccharides, including those having diverse structural moieties or functional groups appended to the core saccharide residues. However, common assays for reducing ends, specific sugar moieties (such as uronic acid residues in this work) or other characteristics of these sugars are often employed quantitatively without elucidating the potential effect of unnatural chemical modification and/or differences in the appended moieties on signal intensity. To this end, the results of this study more broadly emphasize that structural modifications to a polysaccharide can have a significant and variable effect on quantitative detection assays even when the structural modifications are simply differences in the structure of appended groups or other

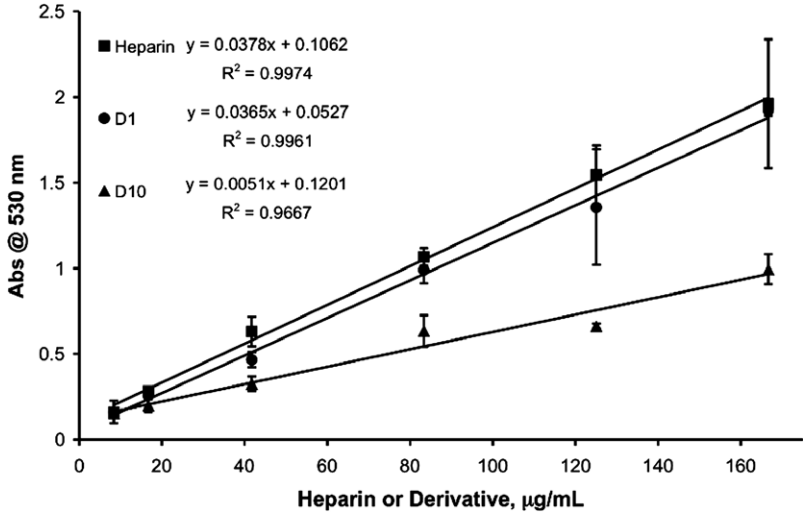


Figure 5. Comparison of standard curves for heparin and two N-desulfonated/N-acylated heparin derivatives. (■) Heparin; (●) 100% N-desulfonated/N-acetylated heparin, **D1**; (▲) 100% N-desulfonated/N-acylated heparin, **D10**.

modifications that do not involve residues or functionality involved in the chemical mechanism of a given analytical assay.

1. Experimental

1.1. Materials

Carbazole was from Alfa Aesar (Ward Hill, MA) and recrystallized from toluene before use.[8,13] Removal of impurities in the carbazole was assessed by TLC (hexanes–Et_2O, 19:1).[14] Concentrated H_2SO_4 (95–98%, density 1.84) ACS reagent was from Sigma–Aldrich (St. Louis, MO). $Na_2B_4O_7{\cdot}10H_2O$ certified ACS from Fisher Scientific (Hampton, NH), was ground to a powder before dissolving in H_2SO_4. All H_2O solutions were prepared using deionized, glass-distilled H_2O from a Corning mega-pure distiller (Corning, NY). Absolute ethanol was purchased from Aaper Alcohol and Chemical Company (Shelbyville, KY).

1.2. General methods

1.2.1. Heparin and heparin derivatives. Heparin, from porcine intestinal mucosa, was from Celsus Laboratories, Inc (Cincinnati, Ohio). All heparin derivatives employed in this study were prepared from the same lot of heparin. Synthesis and characterization of all *N*-acyl heparin derivatives employed in this work has been previously reported.[11,12] Structures of the heparin derivatives employed here, having *N*-sulfo groups replaced with *N*-acyl moieties, are shown (Table 1; **D1**–**D10**). The percent N-desulfonation/N-acylation for individual derivatives other than complete replacement of all *N*-sulfo groups (100% N-desulfonation/N-acylation) is denoted in the manuscript as the number before compound label (e.g., **12D10** indicating *N*-acyl group **D10** replacing 12% of *N*-sulfo groups). Percent N-desulfonation/N-acylation of compounds employed in this work were 12, 21, 33, 47, and 100.[11,12]

The heparin employed here is composed of 70% *N*-sulfo- and 30% *N*-acetyl glucosamine residues (see Fig. 1), $MW_{ave} = 15{,}000$. Molar concentrations of samples were calculated and compared using disaccharide-averaged molecular weights; 660 g/mol was assigned for unmodified heparin. For 100% N-desulfonated/N-acylated samples, 103 g/mol was subtracted for removal of *N*-sulfo groups and sodium from 70% of the disaccharides, to which the MW of each *N*-acyl group was used to calculate and add *N*-acyl contribution to MW of the 70% N-desulfonated disaccharides. Molecular weights for partially N-desulfonated heparin were similarly calculated using the percent *N*-sulfo groups replaced relative to 100% N-desulfonation removing *N*-sulfo groups from the 70% of heparin disaccharides bearing *N*-sulfo groups.

1.2.2. General procedure for the carbazole assay. The carbazole assay was performed in 96-well plates essentially as described by Volpi and co-workers.[7] Briefly, stock solutions were prepared by accurate weighing of samples for each test polysaccharide on an analytical balance followed by dissolving in H_2O to 1 mg/mL. Single concentration comparisons employed 25 µg/well sample in each well unless otherwise indicated. Serial dilution was used to prepare 0.75, 0.5, 0.25, 0.1, and 0.05 µg/µL sample solutions. Fifty microliter of each solution was added to a well of a costar® 96-well 3599 plate from Corning Inc. (Corning, NY). Alternatively, 2.5, 5, 12.5, 25, 37.5, or 50 µL of the 1 mg/mL stock

solutions were transferred to wells and adjusted to 50 μL with H_2O.

To each sample was added 200 μL of concd H_2SO_4 containing 25 mM $Na_2B_4O_7{\cdot}10H_2O$. Plates were heated for 10 min at 100 °C, cooled at room temperature for 15 min, then 50 μl of 0.125% (1.25 g/L) carbazole in absolute ethanol added. Plates were heated at 100 °C for 10 min, cooled at room temperature for 15 min, and then read at 530 nm on a Spectra Max 190 96-well plate reader (molecular devices). Baseline/sample controls included wells with concd H_2SO_4 (with or without borate) with H_2O only, no test sample. Carbazole-free ethanol was employed for experiments as reported with data. Average absorbance data is reported for two separately weighted test samples, each analyzed in triplicate.

Absorbance intensities in this work were significantly increased over intensities reported in the previously described 96-well plate-based carbazole assay. For comparison purposes, a 50 μg test solution of heparin per well yielded an absorbance of 0.4 at 550 nm, as reported by Volpi, while 50 μg of heparin in this work gave an absorbance of 1.7 at 550 nm. It is unclear if this dramatic increase in signal intensity was due to our use of recrystallized carbazole, due to differences in plate readers, or a combination of both.[7]

Acknowledgements

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