Colorimetric quantification of amino groups in linear and dendritic structures

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Abstract

BACKGROUND: The aim of the work reported was to develop a procedure using 96-well microtiter plates for the easy determination of protonated groups of compounds including linear poly(amino acid)s and dendritic polymers divided into dendrigraft and dendrimeric structures. This study is a prerequisite step for the quantification of protonated groups in a macromolecule grafted onto a solid surface.

RESULTS: The procedure was developed from the modified Bradford protein assay and incorporates several modifications that enable one to determine available amino groups (or even other cationic groups) present on the polyresidues backbone, all within five minutes. Based on the Atherton mathematical model, we evaluated the maximal number of Coomassie blue binding sites on linear, dendrigraft or even dendrimeric structures.

CONCLUSION: The mean calculated percentage of occupied sites on a given macromolecule led us to demonstrate that one Coomassie blue molecule interacts with only one single protonated group. Consequently, the developed method using Coomassie blue binding can be used for the quantification of cationic groups in a macromolecule grafted onto a solid surface.

Keywords: Coomassie blue; amino group quantification; poly(amino acid)s; dendrimer; dendrigrafted lysine

INTRODUCTION

The covalent immobilization of active macromolecules onto solid surfaces is of great interest in various fields including biomedical analysis, packaging technologies used for drug extraction, cell culture, catalytic processes, antimicrobial filters and miniaturized devices.1-4 To ensure the performance of the active surface, a characterization step is required. The work reported in the present paper is a prerequisite step for the quantification of amino groups (or even other cationic groups) present on an active surface. The paper presents an extension of a well-known method for the in-solution quantification of amino groups present in biological macromolecules like amino acid polymers and their derivatives.

To date, α-amino acid polymers have been much studied and developed for biomedical applications. Indeed, these structures have been reported to be biodegradable, water soluble and of low toxicity. As they constitute protein mimics, poly(amino acid)s have a considerable potential as carriers for drug delivery, and are particularly useful for the delivery of active substances. In addition, they bear multiple functional groups on their side chains, which can be chemically modified for pharmaceutical agent attachment. However, their characterization based on the determination of the number of available functional groups is not easy since methods for in-solution quantification are mainly based on the derivatization of lysine groups in the presence of labelling probes, which requires proper choice of buffers and reaction times. In order to evaluate precisely the amino (or other protonated) group content of poly(amino acids) and polymeric structures, a quantification method based on the Atherton mathematical model5 and the interaction of the Coomassie Brilliant Blue G-250 (CBB) dye with available protonated groups is presented in this paper. Coomassie blue is well known as the colorimetric component of the Bradford assay commonly used as routine in laboratories for sensitive quantitation of proteins.6

Our aim was firstly to determine whether binding of the dye could occur with linear poly(amino acid) structures having lysine, arginine, proline and histidine residues, and secondly to extend the procedure to a novel class of amine-functionalized macromolecules, which have a large number of branching points and end groups, in order to quantify the number of protonated groups available on the structures including dendrimer, hyperbranched or dendrigraft derivatives. Indeed, due to their globular and more or less compact structure with many functional end groups, dendrimeric and dendrigraft compounds have been reported as having unique properties differing from those of conventional linear macromolecules.7

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For example, dendrimers have been investigated as carriers for controlled drug delivery or have been used as antimicrobial reagents.\(^7\)\(^8\) Nevertheless, the application field of dendrimers is still growing slowly, as there is a major drawback: the characterization step resorts to combined physicochemical methods in order to determine precisely the total number of functional groups at the periphery of the structures. The evaluation of dendrimer or dendrigraft structure properties cannot be easily conducted without knowing the number of their available surface end groups, as this information can be vital to proper interpretation of experiments, in which, for example, dendrimers are considered as carriers by bearing covalently bound pharmaceutical agents.

Consequently, our third goal was to establish a quantification method allowing the determination of the available end groups, being in the present study in their protonated form. Our work focuses on poly(L-lysine) (poly(Lys)), poly(L-arginine) (poly(Arg)), poly(L-histidine) (poly(His)) and poly(L-proline) (poly(Pro)) as linear macromolecules. Dendrigraft structures are represented by five generations of a synthesized family of compounds called dendrigraft of lysine (DGL), for which physicochemical characterization has been recently reported.\(^9\) Finally, polyamidoamine (PAMAM) and poly(ethylene imine) (PEI) backbones were chosen, respectively, as representatives of dendrimer and hyperbranched classes of polymeric amines (Fig. 1).

**EXPERIMENTAL**

**Materials**

Commercial Bradford assay (Coo Protein assay UPF8640) and standard bovine serum albumin (BSA) were products of the Uptima Nunc Company. CBB was provided by Sigma (B5133, \(\geq 90\%\) of purified dye). All other solvents and chemicals were of the analytical grade available. Poly(L-lysine hydrobromide), poly(L-arginine hydrochloride), poly(Pro), poly(His) and generation 5 of PAMAM dendrimer having an ethylene diamine core (5 wt% solution in methanol) were purchased from Sigma. Generations 1, 2, 3, 4 and 5 of DGL were kindly provided by COLCOM Company (France). PEI products having a polymer sequence of \(-\text{(CH}_2-\text{CH}_2-\text{NH})_n\)- with \(10 < n < 10^5\) were a gift from BASF Company (Germany). Three PEI products of weight-average molecular weights \(M_w\) of 5000 g mol\(^{-1}\) (Lupasol G100\(^{\text{TM}}\), 50% in water), 25 000 g mol\(^{-1}\) (Lupasol G500\(^{\text{TM}}\), 40% in water) and 750 000 g mol\(^{-1}\) (Lupasol P\(^{\text{TM}}\), 50% in water) were chosen as unknowns for the study. PEI and PAMAM concentration rates in solution were taken into account for the determination of binding sites.

**Instrumentation**

All spectrophotometric measurements were recorded with an Apollo LB911\(^{\text{TM}}\) absorbance microplate reader from Berthold Technologies having a wide measurement range up to an optical density (OD) of 3.6. Assays were run in Maxisorp\(^{\text{TM}}\) microplate from Nunc Company.  

**Figure 1.** (a) Structure of CBB. (b) Partial structural formula of PEI. (c) Structure of an amine-terminated G2 PAMAM dendrimer with an ethylenediamine core. (d) Structure of the second generation of DGL, synthesised from DGL(G1) consisting of a polypeptide of eight lysine residues.
Preparation of standard solutions

Stock solutions of BSA, poly(Lys), poly(Arg), poly(Pro), poly(His), DGL (generations 1, 2, 3, 4, 5), PEI and PAMAM dendrimers were prepared at 100 µg mL⁻¹ in water (18 MΩ) purified from a MilliQ UV Plus Millipore system. Standard solutions were all freshly prepared from the above stock solutions avoiding successive dilutions. Water mixtures of the poly(amino acids) or other macromolecules were at a final concentration of 0.2 to 40 µg mL⁻¹ when processing the microplate modified Bradford assay as explained in the following section.

Microplate procedure

Assays were carried out in 96 native microplate format wells as described by the supplier. The dye reagent was added to the stock macromolecule solution containing protein, linear or dendritic structures so that the dye concentration (D_T) was maintained constant in 300 µL final volume per well with 1:1 CBB solvent:water mixture volume. After mixing the solution and waiting for 5 min in order to allow maximum dye binding, the absorbance was monitored at 620 nm. All experiments were repeated at least three times per sample concentration. When adding a macromolecule mixture to the dye solution, the observed change in absorbance (ΔA) is then given by the following equation based on the Lambert–Beer law:

\[ \Delta A = (a_{\text{Bound}} - a_{\text{Free}})D_{\text{Bound}} \]  

where \(a_{\text{Bound}}\) and \(a_{\text{Free}}\) are species molar absorptivities for bound and free dye under the microplate assay conditions with pH being about 1, \(D_{\text{Bound}}\) is the concentration of bound dye and \(l\) is the optical length path.

Determination of molar absorptivities

To optimize binding of selected macromolecule sites with dye, we carried out experiments at various dye/macromolecule ratios. We prepared a modified Bradford reagent following a procedure reported by Peterson. Briefly, CBB (20.5 mg) was dissolved in 95% ethanol (10 mL) followed by the addition of 85% phosphoric acid (20 mL). This mixture was completed with ultrapure water to 120 mL and sonicated. Solutions containing dye concentrations between \(2 \times 10^{-5}\) and \(2 \times 10^{-4}\) mol L⁻¹ were prepared from the modified reagent. Free anion dye molar absorptivity \((a_{\text{Free}})\) was estimated from a Beer’s law plot considering an optical path length \(l\) of 1.05 cm (well volume solution high). The \(a_{\text{Free}}\) value was 14 199 L mol⁻¹ cm⁻¹. The obtained data were processed using a simplified Atherton mathematical model considering that only CBB blue species are taken into account during binding so that \(a_{\text{CBB blue species}} = a_{\text{Bound}}\). A double reciprocal plot representation was drawn to provide a value of the absorptivity factor \((\Delta a)\) and molar absorptivities of macromolecule-bound dye \((a_{\text{Bound}})\). Molar absorptivities were calculated under excess macromolecule conditions.

Calculations of binding sites (n)

The number of binding sites can be determined according to the Atherton mathematical model and considering the two following experimental conditions.

1. If excess macromolecule conditions prevailed, all dye moieties were considered to be bound \((D_{\text{Bound}})\) to macromolecule sites, so that \(D_{\text{Bound}} = D_T\). In that case, only strong binding sites are active in binding dye \((n)\) becomes \(n_{\text{strong}}\). Equation (1) becomes

\[ \Delta A = (a_{\text{Bound}} - a_{\text{Free}})D_{\text{Bound}} = (\Delta a)D_T \]  

The number of strong binding sites \((n_{\text{strong}})\) can be calculated from the following equation using the slope of the assay plot:

\[ n_{\text{strong}} = \frac{(\text{slope of assay (µg}^{-1})\times (MW) \times \text{(dilution coefficient)}}{\Delta a} \]  

where MW is the molecular weight of the macromolecule considered.

2. Under conditions of excess dye, which favour binding saturation, a maximum number of the macromolecule binding sites should be covered by the dye \((n = n_{\text{total}})\); then \(D_T \gg n_{\text{total}}P_T\). This leads to \(D_{\text{Bound}} = n_{\text{total}}P_T\), where \(P_T\) is defined as the molar macromolecule concentration. Substitution of \(D_{\text{Bound}}\) for \(n_{\text{total}}P_T\) in Eqn (1) gives

\[ \Delta A = (\Delta a)ln(n_{\text{total}}P_T) \]  

The value of \(n_{\text{total}}\) was estimated under excess dye from the slope of a plot of \((\Delta A)\) versus \(P_T\) as long as the plot is linear.

RESULTS AND DISCUSSION

In characterizing a carrier compound for drug delivery applications, knowledge of the number of functional groups available on its structure is required as it may increase the drug crosslinking rate and have a dramatic effect on the sensitivity of the experiments. As the colour intensity of the Bradford assay and protein-to-protein variability were reported to be highly dependant on protein composition, we first evaluated the ability of CBB to bind linear poly(amino acid) residues. This way of quantification seemed to be plausible as the protonated side chains of the arginine and lysine residues have been shown to be essential for CBB bindings on protein structures. Second, we studied the CBB binding on aminated dendrigrift and dendrimeric structures. For this, we used a commercially available 96-well microtitre plate Bradford assay.

Running microplate Bradford assays

Spectrophotometric analyses were performed at 620 nm and samples were prepared as described above using BSA as the standard in the microplate procedure. The resulting absorbance changes occurring when adding increasing quantities of stock poly(amino acid) solution are illustrated in Fig. 2(a). Absorbance change is calculated as the difference between the observed CBB–macromolecule complex absorbance and that of the initial dye concentration (no macromolecule present) and refers to Eqn (1) based on Beer’s law and describing the macromolecule–dye binding process. Except for poly(Pro), increasing concentration of macromolecule induces an absorbance change increase, proving that dye is able to bind to histidine, arginine or lysine residues but has no ability to bind to proline residue, which is in agreement with previous work. In contrast to the BSA results, the assay curves obtained with cationic poly(amino acid)s do not fit straight lines under the assay conditions but markedly deviate from linearity at concentrations above 10 µg mL⁻¹. As reported elsewhere, this nonlinearity is supposed to be due
to the presence of two charge forms of the dye at the pH of the assay and also to a rapid decrease of the free dye in the assay, leading in our case to less dye–poly(amino acid) complex formation. A mechanism of CBB binding to proteins was recently proposed by Georgiou et al.\textsuperscript{18} Those authors suggest that absorbance of the CBB–macromolecule complex is exclusively related to the number of bound dye moieties. This point is in agreement with our results explained in the following subsection of this paper.

The linear regression lines can be calculated only in the narrow range of 2–10 µg mL\(^{-1}\) for poly(His), poly(Arg) and poly(Lys). As expected, the greatest responses were for poly(His) and poly(Arg), and the lowest for poly(Lys). Indeed, the importance of protonated groups (especially lysine and arginine residues) in the binding of CBB has been underlined by many authors.\textsuperscript{13–16} but binding can also be due to the combination of electrostatic and hydrophobic interactions or even to only hydrophobic interactions, as demonstrated by Compton and Jones when studying CBB interactions with poly(amino acids) having functional groups such as histidine, tyrosine or phenylalanine residues.\textsuperscript{11} Moreover, our data confirm that CBB interactions seem to be in favour of positively charged amino acids, since poly(Pro) produced no dye response.

As previously commented for linear poly(amino acids), studies with dendrigraft and dendrimeric structures (Fig. 1) yield saturation binding curve shapes (Fig. 2(b)). Figure 2(b) shows important absorbance changes when adding CBB to DGL and PAMAM structures in water solutions. These results demonstrate the ability of CBB to bind to cationic globular and macromolecular PAMAM structures or even spherical polyamines (PEI), which have a well-defined ratio of primary, secondary and tertiary amine functions (Figs 1 and 3). Our data are consistent with previous studies of the electrophoretic separation of amine-terminated PAMAM using CBB during the staining step.\textsuperscript{11,12} These results are in accordance with our previous remark about CBB interactions with protonated end groups other than those of lysine and arginine side chains. All the selected structures have effectively various protonated end groups (guanidino groups, aromatic heterocyclic amine, imine, etc.) proving at the same time the feasibility of our CBB approach, which could be further extended to other dendritic, hyperbranched compounds having other types of protonated end groups.

**Saturation effects with Bradford assay**

Considering the shortened linear domain of dendritic and dendrigraft compound assay curves, we hypothesized that there is an insufficient quantity of dye in the commercial assay reagent, which minimizes the dye-binding process. This is in agreement with the findings of Read and Northcote, who demonstrate that the saturation effect would most likely be related to a saturation in the dye-binding process.\textsuperscript{12} To emphasize this point, we evaluated the curve shape tendency of the assay by comparing spiked samples and standard solutions of poly(Lys). It can be seen from Fig. 3(a) that the curve shape for 1.25 × 10\(^{-4}\) mol L\(^{-1}\) dramatically turns into a saturation line (\(×\)) after addition of a known quantity of poly(Pro) in the assay. This saturation effect can be explained by an excess of poly(amino acids) in the spiked sample, and all of the CBB is bound to the protonated sites. This suggests that the number of bound dye moieties influences the absorbance of the CBB–macromolecule complex as described by Georgiou et al.\textsuperscript{18} This also means that the linear range should be improved in order to quantify these macromolecular structures (except BSA) using CBB leading to a modified Bradford assay.

In conclusion, we have demonstrated the ability of CBB to bind all of the tested amine-functionalized structures. To quantify the number of CBB binding sites on our macromolecules, the method of quantification based on the Atherton mathematical model has been adapted. This model requires two steps. The first step consists of measuring the molar absorptivity of the bound dye (\(\varepsilon_{\text{Bound}}\)) under excess macromolecule conditions. The second step consists of calculating the total number of CBB binding sites per cationic molecule (\(n_{\text{Total}}\)) for a high dye concentration (\(D_{\text{T}}\)) over the macromolecule concentration range. Thus, we will be able to determine a correlation between macromolecule–dye complex formation and the amount of available protonated groups of the molecule of interest.

**Determining molar absorptivities of macromolecule-bound dye**

To determine molar absorptivities of macromolecule-bound dye (\(\varepsilon_{\text{Bound}}\)), assays should be done at a high macromolecule content to dye ratio. For that particular condition, all dye moieties are considered to be bound (\(D_{\text{Bound}}\)) to macromolecule sites, so that \(D_{\text{Bound}}\) is equal to the initial concentration of the dye (\(D_{\text{T}}\)) and only binding sites (\(n_{\text{Bound}}\)) are active in binding dye. In the research described herein, optimization of the dye concentration was required for amine or other protonated group determination.

Following a published modified Bradford protocol,\textsuperscript{6} we prepared a series of dye reagent concentrations (\(D_{\text{T}}\)) in order to measure the molar absorptivity of the bound dye. For instance, while quantitative analysis of BSA can be easily done at

![](image-url)
2.1 \times 10^{-5} \text{ mol L}^{-1} \text{ with a linear domain of 4–25 \mu g mL}^{-1}, \text{ the assay plots of all others macromolecules including poly(Lys), dendrigraft and dendrimeric structures cannot be used at this concentration as the saturation effect occurs very quickly (Fig. 3). It is then not possible to draw reciprocal plots for the determination of maximal variation of absorbance } \Delta A_{\text{max}} \text{, which leads to the calculation of } \delta_{\text{bound}}. \text{ In addition, DGL(G1) did not induce an absorbance change with a dye concentration under } 0.8 \times 10^{-4} \text{ mol L}^{-1}, \text{ indicating that the dye binding process is less important than with other tested cationic compounds. This could be explained by the low molecular weight of DGL(G1) (1400 g mol}^{-1}, \text{ as its structure is linear and has an average composition of eight lysine residues. This result correlates well with the work of Sedmark and Grossberg, demonstrating that peptides with a molecular mass of less than 3000 g mol}^{-1} \text{ lead to poor complex formation.}^{15} 

For all the samples studied in this paper, we then chose to calculate molar absorptivities at a dye concentration of \(0.8 \times 10^{-4} \text{ mol L}^{-1}\) in order to stay in the conditions of high protein to dye ratio, except for DGL(G1), for which calculations were done at \(1.25 \times 10^{-4} \text{ mol L}^{-1}\), and for BSA and poly(Arg), for which calculations can be done at \(0.6 \times 10^{-4} \text{ mol L}^{-1}\). Corresponding double reciprocal plots of reciprocal absorbance change at 620 nm versus reciprocal macromolecule concentration at the selected constant dye concentration are shown in Fig. 4. Values of \(\Delta A, \Delta a\) and \(\delta_{\text{bound}}\) can be found from the reciprocals of the intercepts of these plots and are listed in Table 1. The molar absorptivities of the
bound dye depend on the tested structures to which it is bound as they vary from 22 000 to 70 000 L mol$^{-1}$ cm$^{-1}$. Molar absorptivities of DGL, PEI and PAMAM are around 30 000 L mol$^{-1}$ cm$^{-1}$, which is consistent with the value obtained for poly(Lys). Under normal Bradford assay conditions, $\epsilon_{\text{free}}$ was reported to be about 11 000 L mol$^{-1}$ cm$^{-1}$, while for many proteins $\epsilon_{\text{bound}}$ was determined in the range 50 000–60 000 L mol$^{-1}$ cm$^{-1}$. These differences are due to the volume ratio and the selected dye concentration. $D_T$ being taken above the usual assay concentration of 1.33 $\times$ 10$^{-5}$ mol L$^{-1}$, $\Delta A$ was found to be greater. The molar absorptivity differences ($\Delta \alpha$) reported in Table 1 are nevertheless in agreement with the assay sensitivity showing that poly(Arg) and poly(His) exhibit the largest slopes, followed by BSA and DGL(G3), as for under commercial assay conditions. At a dye concentration of 0.8 $\times$ 10$^{-4}$ mol L$^{-1}$, we evaluated $n_{\text{strong}}$ values according to Eqn (3). The data are reported in Table 2, from which a comparison can be made of $n_{\text{strong}}$ with the theoretical total protonated groups on the poly(amino acids), dendrigraft or dendritic structure.

### Estimation of the number of binding sites ($n_{\text{Total}}$) and available amines

The previously reported strategy to estimate the total number of CBB binding sites ($n_{\text{Total}}$) involves high dye/macromolecule ratios; calculations can be done using Eqn (4). We explored the linearity of the assay response with progressively increasing $D_T$ in order to cover the range from 0.8 $\times$ 10$^{-4}$ mol L$^{-1}$ up to instrumental saturation. A minimum of eight macromolecule concentrations within the range 5–25 µg mL$^{-1}$ were prepared for each assay. Linearity can be enhanced by maximizing the dye concentration in the assay mixture as shown in Fig. 3. Each plotted value represents the mean $\pm$ standard deviation (SD) of three replicate experiments. In most cases, a wider range of linear response curves was obtained at 1.6 $\times$ 10$^{-4}$ mol L$^{-1}$ for BSA and poly(amino acids) and at 1.25 $\times$ 10$^{-4}$ mol L$^{-1}$ for DGL and others dendritic structures. Nevertheless, at a concentration of 1.6 $\times$ 10$^{-4}$ mol L$^{-1}$, the results exhibit a much higher SD value, indicating a higher variability in the assay. In addition, assays show a lower sensitivity, whatever the compound tested. Thus, we decided to consider 1.25 $\times$ 10$^{-4}$ mol L$^{-1}$ for the determination of maximal CBB binding sites ($n_{\text{Total}}$) under our assay conditions.

Table 1 summarizes the results obtained using two values of $D_T$. The percentages of occupied sites on the selected compounds are all lower than 100%, suggesting that the ratio of CBB to amine binding is 1:1. Our results are in agreement with previous studies hypothesizing that CBB is able to interact with one single binding site at a time under normal Bradford assay conditions.

As can be seen from Table 2, increasing $D_T$ from 0.8 $\times$ 10$^{-4}$ to 1.25 $\times$ 10$^{-4}$ mol L$^{-1}$ does not increase the number of CBB binding sites on BSA and poly(Arg). This means that when $D_T$ is 0.8 $\times$ 10$^{-4}$ mol L$^{-1}$, conditions of high dye to macromolecule ratios are fulfilled and Eqn (4) can be used. Consequently, for BSA and poly(Arg), $n_{\text{strong}}$ already equals $n_{\text{Total}}$ at $D_T$ = 0.8 $\times$ 10$^{-4}$ mol L$^{-1}$.
This correlates well with the fact that the BSA and poly(Arg) curves remain linear with similar slopes from 5 to 20 μg mL\(^{-1}\) no matter what the \(D_T\) value, as long as it is above 0.6 × 10\(^{-4}\) mol L\(^{-1}\) (Figs 3(b) and (c)). Considering the observation of Congdon et al. that, under the conditions of normal assay, arginine and lysine are mostly active in binding dye,\(^{13}\) our results are in agreement with previous published data, with more than 93% of occupied sites on BSA and poly(Lys). Nevertheless, only 34.4% of CBG is bound to poly(Arg). To a lesser extent, poly(His) is also able to bind dye under our assay conditions.

Lysozyme-containing compounds (poly(Lys) and DGL) and PAMAM dendrimer show a maximal CBG binding at \(D_T = 1.25 \times 10^{-4}\) mol L\(^{-1}\), as no significant differences were observed with assays carried out at \(D_T = 1.4 \times 10^{-4}\) mol L\(^{-1}\) (data not shown). The percentage of occupied sites is above 90% for DGL(G2), DGL(G3) and PAMAM(G5), whereas it decreases to 74.8% for DGL(G5), indicating that its primary amines are less accessible to interact with CBG than the other cationic structures. The slight difference in the determined value of \(n_{\text{Total}}\) between PAMAM(G5) and DGL(G3), despite both having similar molecular weights and total number of amino groups, can be explained by their architecture. The dendrimer has a well-controlled compact architecture, built step by step from its core, whereas the dendrigrift structure is less controlled with grafting initiated from a linear polymer that constitutes the heart of successive generations. Consequently, when branching occurs from one generation of dendrigrift to another, amino groups can be left free since the branching ratio may be less than 100%. This means that among the average amine content of 123 of DGL(G3),\(^{9}\) some groups are located within the core so that CBG might not be able to associate with them. In contrast, PAMAM does not contain free amino groups within its core, but its amino groups are only located at its surface that might favour interaction with CBG. Another explanation for the observed difference in binding CBG could be the more flexible structure of DGL (resulting from branching from a segment) than the dendrimeric structure (Fig. 1). Indeed, the more extended and flexible structure of DGL may undergo uncontrolled space constraints such as chain folding towards its core and steric hindrance that can reduce accessibility to CBG molecules. This last comment seems to be consistent with the results for DGL(G4) and DGL(G5) as the percentage of occupied sites is lower when increasing from one generation of DGL to another (Table 2). Nevertheless, our data suggest that to have about 120 available amino sites, only a third generation of DGL is required whereas a fifth generation of PAMAM is needed. Indeed, if we hypothesized that 90% of sites could be covered on PAMAM(G3), the value of \(n_{\text{Total}}\) would be 29, whereas the calculated \(n_{\text{Total}}\) value for DGL(G3) is 114 (Table 2).

Available binding sites were also determined for hyperbranched PEI structures. Possible protonation sites on PEI structures have not been clearly defined by the manufacturer. From both the molecular weight of PEI and the primary/secondary/tertiary amine ratios given by the technical information sheet provided by BASF, we have calculated that Lupasol P100, Lupasol G500, and Lupasol P have, respectively, ratios of 43:43:30, 207:228:159 and 6443:6443:4510 (primary:secondary:tertiary). The number of binding sites \(n_{\text{Total}}\) reported in Table 2 leads us to hypothesize that either primary and secondary amines but not tertiary amines are able to interact with CBG, or all amino groups (primary, secondary, tertiary) are able to interact with CBG but not in a quantitative way probably due to steric hindrance. This second hypothesis seems to be compatible with the results obtained for Lupasol P, which has a small value of \(n_{\text{Total}}\).

Our CBG method is, then, convenient as it allows a comparison of available sites between aminated compounds, and might be a means to guide further researcher choice before conjugation of pharmaceutical entities.

Finally, from all of the data presented in this paper, the accurate determination of the number of CBG binding sites (\(n\)) on macromolecules can be divided into three key steps. Step 1 consists of running a commercially available Bradford assay taking macromolecule concentration within the range 5–25 μg mL\(^{-1}\). Step 2 is the determination of molar absorptivities of macromolecule-bound dye (\(a_{\text{Bound}}\)). The choice of \(D_T\) is made according to the assay responses observed in step 1. Two cases can be distinguished before proceeding to step 2:

- If the assay curve obtained fits a single straight line in the selected concentration range, then \(a_{\text{Bound}}\) should be determined using a \(D_T\) value of 0.6 × 10\(^{-4}\) mol L\(^{-1}\).
- If the first steps leads to a saturation curve shape, \(a_{\text{Bound}}\) should be calculated at a dye concentration of 0.8 × 10\(^{-4}\) mol L\(^{-1}\).

During step 3, taking \(D_T\) as 1.25 × 10\(^{-4}\) mol L\(^{-1}\) allows the accurate determination of maximal number of CBG binding sites (\(n_{\text{Total}}\) on macromolecules. All experiments are done with macromolecule concentrations ranging from 5 to 25 μg mL\(^{-1}\). In addition, if the theoretical total number of cationic groups was previously determined by another and independent analytical method, then the percentage of occupied sites per macromolecule can be calculated. The calculated percentage of occupied sites led us to demonstrate that a CBG molecule interacts with only one single protonated group.

**CONCLUSIONS**

We have developed a microplate procedure which relies on the interaction of a colorimetric reagent, CBG, with protonated groups available on linear poly(amino acid), dendrigraft or dendrimeric structures following a mathematical model previously described by Atherton and co-workers. At a \(D_T\) value of 1.25 × 10\(^{-4}\) mol L\(^{-1}\), the sensitivity of the assay was sufficient to evaluate the maximal number of CBG binding sites (\(n_{\text{Total}}\)) for all the selected cationic structures (linear or not). The value of \(n_{\text{Total}}\) was found to correlate well with the number of available protonated groups of the tested structures. Moreover, through the example of PAMAM dendrimers and PEI compounds, we have demonstrated that CBG is able to bind without specificity to macroglular structures having various protonated end groups. As a consequence, this procedure might be applied to evaluate available amino groups (or other cationic groups) present on future synthesized dendrigraft, dendrimer or even hyperbranched polymeric structures. Knowing \(n_{\text{Total}}\) might also be helpful to estimate moiety attachment rate after a conjugation step on a dendritic or linear structure. Finally, knowing that CBG interacts with one single protonated group, our next paper will describe the quantification of amino groups bonded on various solid surfaces.\(^{20}\)

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