CHOICE OF PARAMETERS FOR TOLUIDINE BLUE *O* COLORIMETRIC ASSAY: INFLUENCE ON THE DETERMINATION OF GRAFTING DENSITY OF ANIONIC GROUPS**

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The grafting of specific functional groups has been drawing attention later as a simple and effective way to modify materials surfaces to induce a particular response without significant changes in bulk properties and even very small impact in some surface properties. In the case of anionic group grafting, colorimetry analyses using complexed cationic stains are a fast way to determine the grafting density. This technique is especially interesting for the quantification of grafted species such as sulfonates or carboxylate groups. Although simple, this method involves a certain number of crucial parameters and small deviations could lead to unreliable quantification and consequently lead to a performance below the expected. To evaluate how the parameter choice, or errors, could influence the final quantification, poly ε-caprolactone samples were grafted with poly(sodium 4-styrene sulfonate) and stained with toluidine blue O in different conditions of pH, incubation, and decomplexation time, and light exposure. All four studied parameters have shown a stronger influence on the final measured grafting density value.

Keywords: *toluidine blue O, polymer grafting, grafting quantification.*

ВЫБОР ПАРАМЕТРОВ ДЛЯ КОЛОРИМЕТРИЧЕСКОГО АНАЛИЗА ТОЛУИДИНОВОГО СИНЕГО *О*: ВЛИЯНИЕ НА ОПРЕДЕЛЕНИЕ ПЛОТНОСТИ ПРИВИВКИ АНИОННЫХ ГРУПП

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Прививка конкретных функциональных групп привлекает внимание как простой и эффективный способ модификации поверхностей материалов для получения определенной реакции без значительных изменений объемных свойств и без малейшего воздействия на некоторые свойства поверхности. В случае прививки анионных групп колориметрический анализ с использованием комплексных катионных красителей является быстрым способом определения плотности прививки. Метод применяется для количественного определения привитых видов, таких как сульфонаты или карбоксилатные группы, и включает в себя определенное количество важных параметров, небольшие отклонения приводят к ненадежной количественной оценке и, как следствие, к производительности ниже ожидаемой. Для оценки влияния параметров или ошибки на окончательную количественную оценку образцы поли-капролактона привиты поли-4-стиролсульфонатом натрия и окрашены толуидиновым синим О в различных условиях pH, инкубации и времени декомплексации и светового воздействия. Рассматриваемые параметры оказывают большое влияние на измеренную плотность прививки.

Ключевые слова: *толуидиновый синий О, прививка полимера, количественная оценка прививки.*

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Introduction. The advance of materials science brought to light the distinct properties of two different regions of an engineered material: bulk and surface. Both regions have their own features, provide different properties, offer different manipulation possibilities, and require distinct treatments. To work specifically on the chemical composition of materials surfaces, several techniques have been proposed based on interactions of diverse natures between the modified substrate and the added substance. Nevertheless, a simple physical adsorption or other interaction of electrostatic nature may not be enough to ensure durable effects, and stronger binding techniques, such as covalent grafting, deserve special consideration as resilient modifications [1].

In the biomaterials field, the study of such techniques has attracted significant attention owing to the close interaction between host and implant surfaces [2] and, among the numerous parameters involved in those treatments, the quantification of grafted material is, without doubt, a defining factor.

Over the years, this quantification, usually defined as grafting efficiency, grafting density, or even grafting rate, was performed using a few techniques such as kinetic models [3], weight gain [4], solvent extraction [5], and later, chromatography methods [6], which have been gained popularity over the other techniques owing to the ability to detect a fairly low amount of grafted material with simplicity and accuracy.

A successful example of grafting quantification using chromatography is the complexation of cationic stains over negatively charged groups on a material surface, such as toluidine blue O staining (TB). Initially developed for biological tissue staining owing to its affinity to nucleic acids [7], TB is an organic stain of the phenothiazine family. As a result of its cationic behavior, TB can be easily complexed over anionic groups, especially carboxylate, sulfonates, and phosphates [8]. This property has been successfully exploited to quantify RGD peptides [9], heparin [10], poly(acrylic acid) [11], and poly(sodium 4-styrene sulfonate) [12].

Although simple, this technique should be conducted carefully, as it is composed of several steps, each one with its particularities and strong influence between each other. On grafting experiments, the expected grafting rate is quite low, in the order of micrograms per sample [13, 14]. In applications such as biomaterial modification, the precise evaluation is crucial for the final performance [15], and even a very small fluctuation of the quantification could imply that the surface will present a behavior different from the reality of the final application. With this in mind, this note is aimed at studying the parameter choices for the utilization of this method and shows how small variations in measuring time or conditions could lead to unreliable results.

Experimental. *Chemicals and reagents.* Toluidine blue O staining was bought from Carl Roth (Karlsruhe, Germany), poly ε-caprolactone (PCL, 80 kDa) was bought from Corbion (Amsterdam, Netherlands), and sodium styrene sulfonate (NaSS), glacial acetic acid, and dichloromethane were bought from Sigma-Aldrich (St. Quentin Fallavier, France).

Sample preparation. PCL films were cast by dissolving PCL pellets in dichloromethane (30% w/v). The solution was poured into a glass support and spun in a SPIN150-v3 SPS spin-coater. The films were allowed to dry at room temperature and cut into round samples (diameter 14 mm).

The thermal grafting technique was previously described [16]. Briefly, the surfaces were activated by ozonation (0.5 bar/0.6 L/min flow rate) at 30°C for 20 min before transferring to a balloon containing NaSS solution (0.7 M) and heated at 45°C for 1 h for grafting. Grafted samples were washed in deionized water for 48 h, vacuum dried, and stored at 4°C before experiments.

Toluidine blue O colorimetric assay. Standard conditions for pNaSS grafting density determination were considered as follows [17]. A TB solution (0.5 mM in deionized water) was prepared at room temperature using a volumetric balloon. The solution pH was adjusted to 10 using a minimal volume of NaOH (1 M) and the TB solution was kept away from the light. Each PCL sample was placed in an aluminum foil-wrapped flask containing 5 mL of TB solution and a magnetic stirrer. The flasks were heated to 30°C in a water bath for 6 h. After incubation, the samples were washed 3 times for 5 min under mechanical stirring in NaOH (1mM solution). Afterward, each sample was placed in a flask with acetic acid solution (50% v/v in water), the flasks were wrapped in aluminum foil and kept under mechanical stirring for 24 h for decomplexation. The absorbance of resultant solutions was measured using UV-visible spectroscopy (Perkin 167 Elmer lambda 25 spectrometer; Waltham, MA, USA) at 633 nm and the grafting density (GD) was calculated:

$$\mathrm{GD} = (AV)/(\varepsilon lm), \quad (1)$$

where A is the sample absorbance, V is the acetic acid volume, l is the length of the UV cuvette, m is the mass of the sample, and ε is the molar attenuation coefficient of TB solution. The ε was calculated as an angular coefficient of a curve of the known TB dilutions ranging from 0 to 32.5 μM. Nongrafted samples were subjected to the same process as a negative control.

The following parameters were changed to study their influence on GD determination: calibration curve construction, pH of TB solution, incubation and decomplexation time, light exposure. Whenever one of these parameters was changed all others were set as in the described standard conditions.

Fourier-transform infrared spectroscopy. Fourier transform infra-red spectroscopy in attenuated total reflection mode (ATR; Perkin Elmer Spectrum Two) was performed over grafted and nongrafted samples before and after the TB assay to evaluate the presence of nonspecific complex stain in the surface by the end of the process. A total of 128 scans were performed (4000 to 400 cm^{-1}, resolution 2 cm^{-1}) at three different points per sample.

TB quantification and practical applications. The quantification of grafted groups by TB could indicate how the surfaces will behave during the final application. In the specific case of the pNaSS grafting degree, the BT results could imply a favorable cell adhesion over the samples. To verify that possible samples with different measured grafting degrees were incubated with primary merino sheep fibroblast for 1, 3, and 7 days, as previously described [18]. Once the time point was reached, the samples were rinsed twice in PBS, the cells detached using trypsin-EDTA 0.05 % solution, and counted with a handheld automated cell counter (Scepter 2.0; Millipore, Burlington, MA, USA).

Statistical analysis. Based on previous studies the variation of the measured GD was considered to follow a normal distribution and the minimal statistical sample size (N) was calculated using the equation:

$$N = (Z_{99}\sigma/E)^2, \quad (2)$$

where Z_{99} is the z score for 99% confidence level (2.575), σ is the standard deviation of the GD (6.68×10^{-7} mol/g), and E is the maximum permissible error (10^{-6}).

Statistical differences between the studied conditions were calculated with the ANOVA test and Tukey's pairwise analysis using past 3 software ($\alpha < 0.05$).

Results and discussion. *Calibration curve construction.* The determination of the molar attenuation coefficient is a key step for the accuracy of any colorimetric method. In the case of the TB assay, for a 0.5 mM solution, the standard ε value is 47,006±1250 $L \cdot mol^{-1} \cdot cm^{-1}$. A common mistake in the calibration curve preparation is the choice of the solvent for dilution of the original solution. The TB solution is prepared in deionized water; however, the dilution should be prepared under the same conditions as for the decomplexation, i.e., performed in acetic acid.

Figures 1a and b show the typical curve for these two solvents. The absorbance is calculated using the following equation:

$$A = \lg (I_0/I),$$

where I_0 is the intensity of incident light and I is the intensity of transmitted light.

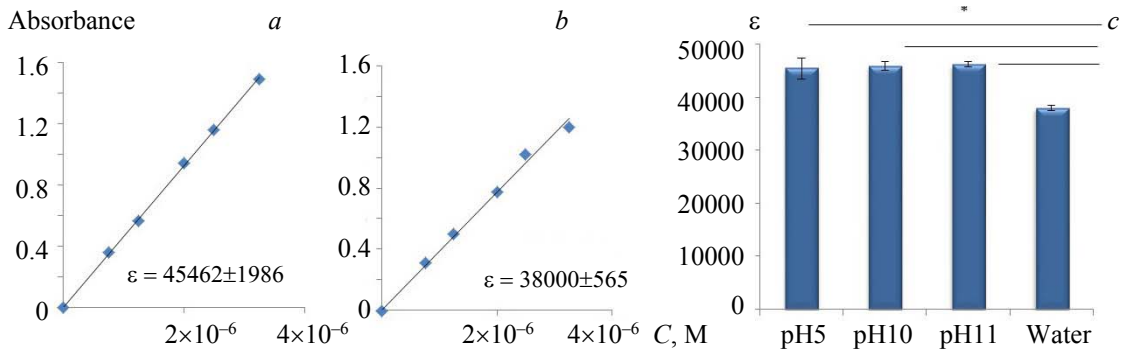

Fig. 1. Typical calibration curve for ε determination in a) acetic acid, and b) deionized water. c) Average ε values for dilutions prepared from a TB solution at a pH of 5, 10, and 11. All conditions diluted in acetic acid presented higher ε ($\alpha < 0.05$) than dilution in deionized water.

The result confirms that dilution in water should be avoided as it led to a significantly ($\alpha < 0.05$) smaller angular coefficient and, considering Eq. (1), would artificially increase the density of sulfonate groups on the surface. As this calibration should be done in an acid solution, the influence of the pH of the TB solution on the ε value is a valid question. To solve that, three different pH values were chosen to evaluate the influence of this parameter on the ε value: the original solution pH ~ 5, the adjusted solution pH 10, and a more

basic pH 11 to simulate an over-adjustment of the pH. Figure 1c shows the average ε values for these conditions. The increase in pH led to a slight increase in the ε value; nevertheless, for all three pH conditions no statistical difference was observed and all values are within the expected range. This result implies that the operator can use a fresh solution to establish the calibration curve even before the pH adjustment.

pH of TB solution for staining. The TB molecule contains two amine groups, which can be deprotonated and complexed on the surface, as seen in the detail of Fig. 2a. The deprotonation of the primary amine could lead to an unbalanced complexation, with one molecule of staining complexing more than one anionic group. To avoid this error and ensure the 1:1 ratio between a mole of TB and a mole of sulfonate on the surface, the staining solution should not have an acidic pH. This phenomenon can be verified by the fairly small GD found for samples stained at pH 5 when compared with the standard pH condition ($\alpha < 0.05$). The experiments conducted under standard conditions (pH 10) presented a GD close to that previously reported for the same substrate [18]; on the other hand, a significantly ($\alpha < 0.05$) higher GD was verified when the pH was increased to 11. However, for the same conditions, nongrafted PCL samples stained and used as negative controls also presented amplified GD values (Fig. 2a, red bar), indicating that the rise for samples stained at pH 11 does not represent the real measure of the sulfonate groups but an increase in nonspecific absorption. The nature of the interaction between surface and staining in the case of nongrafted and grafted surfaces was investigated by FTIR (Fig. 2b). TB adsorbed on nongrafted surfaces was identified by the peak of ν_{as}(N-H) bonding (1538 cm^{-1}). As no pNaSS was present on this surface this interaction is considered to be nonspecific and does not reflect the real grafting rate. Nevertheless, the decrease in the intensity of ν_{as}(N-H) (Fig. 2c) showed the effective release of TB during the decomplexation process, explaining why nongrafted samples can exhibit GD readings. Conversely, the TB adsorption on a grafted surface showed the appearance of two peaks: the same nonspecific absorption peak at 1538 (ν_{as}(N-H)) and a new peak at 1608 cm^{-1} (ν(C-H)) [19] are assumed to be the result of the specific interaction of a TB/grafted surface, by the complexation of $C{=}N^{+}(CH_3)_2$ and $-SO_3^-$ [20, 21]. After decomplexation of the grafted samples, the specific interaction peak completely vanished; yet, the intensity of the nonspecific absorption reveals a negligible fluctuation of nonspecific absorbed groups, an indication that the measured value came primarily from specific absorbed molecules.

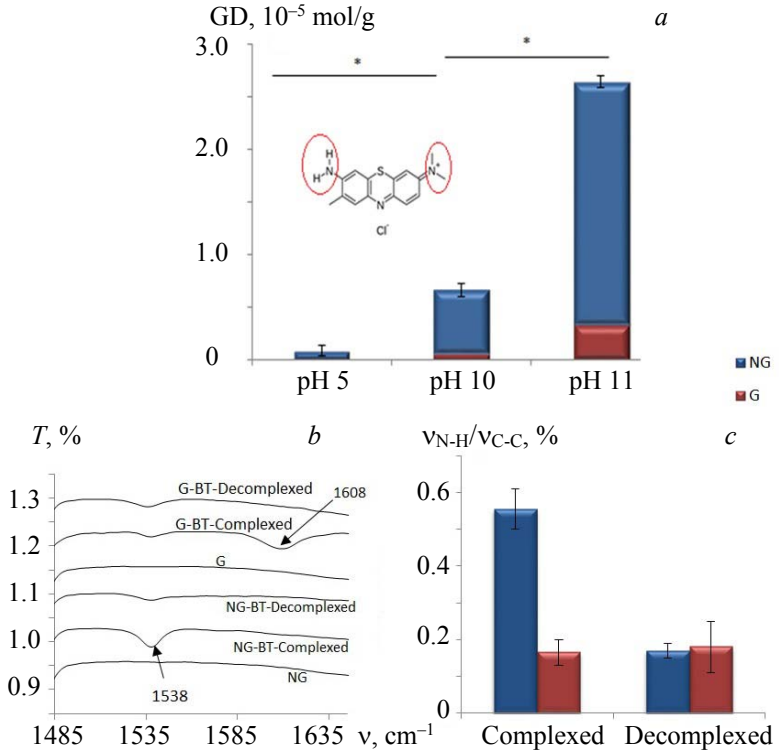

Fig. 2. a) GD of PCL samples when incubated with TB solutions at a pH of 5, 10, and 11. In detail (red circles) two possibly deprotonated amines. b) IR spectra of nongrafted and grafted surfaces by TB complexation and decomplexation. c) Relative intensity of N-H peak before and after decomplexation over grafted and nongrafted samples.

Incubation and decomplexation time. Both complexation and decomplexation are intricate processes and take time to happen. Originally, the complexation time suggested to saturate polymer surfaces with TB was 5 h [22]; nevertheless, our study suggests that more time could be necessary to complete the staining.

As seen in Fig. 3a, samples immersed for 6 h in TB solution have a considerably ($\alpha < 0.05$) greater GD than samples that were stopped at 5 h. On the other hand, the nonspecific absorption over nongrafted samples (red bar) did not show any improvement after 1 additional hour of complexation. Increasing the staining time up to 8 h did not show any significant intensification of the grafting rate measurements. Similarly, Fig. 3b shows the significant ($\alpha < 0.05$) difference between GD of samples that were stopped early and those that were totally decomplexed. Additional experiments conducted after this point (up to 72 h) did not show any relevant increase in GD.

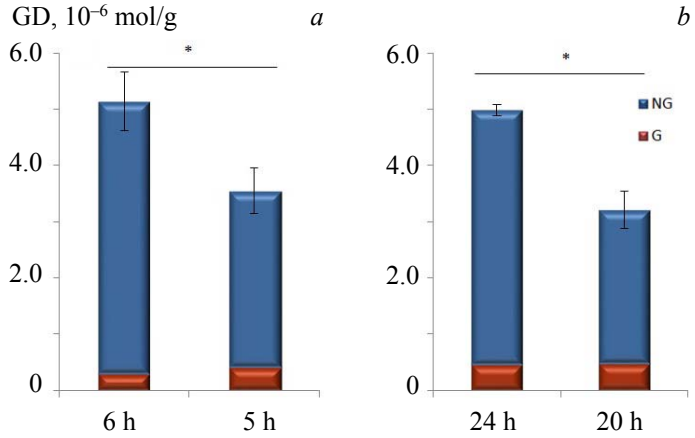

Fig. 3. GD of PCL samples after a) different incubation time, b) different decomplexation time.

Although these timings are not a golden rule for staining and factors such as nature of the subtract and concentration of groups to be measured could interfere in the final result, these results prove that a kinetic experiment of the total staining/decomplexation time should be performed for the studied samples to ensure that the surfaces reach saturation and total decomplexation after each step.

Light exposure and nonspecific absorption. Like many typical stains, TB is photosensitive [23]. To evaluate the impact of the light on the final evaluation of the sulfonate density the staining was conducted under nonprotected conditions and compared with a regular protocol.

Figure 4a shows a significant ($\alpha < 0.05$) reduction of GD for nonprotected samples compared with regular conditions. In fact, the photodegradation of the TB molecule could produce a virtual decrease in the solution concentration, reducing the complexation. Moreover, a small increase in the nonspecific absorption (red bar) was verified, which could indicate that the degraded molecule will present different behavior in contact with the substrate.

TB quantification and practical applications. The presence of pNaSS was reported to improve cell adhesion over several biomaterial–surfaces owing to its favorable interaction with cellular binding proteins [24–27]. An accurate determination of the grafting degree could be a reliable indicator of future cell response. To confirm that, standard conditions of TB assays were performed over PCL films, and samples were divided into three groups of increasing measured grafting degrees. The average GD was measured as follows: nongrafted = 0.3 μmol/g (non-specific absorption), grafted 1 = 1.79 μmol/g and grafted 2 = 2.24 μmol/g.

On the first day, although the average numbers of cells over grafted samples were greater than for nongrafted samples, no statistical difference was verified. This behavior was modified for the subsequent time points. As seen in Fig. 4b, for days 3 and 7, the number of cells followed the grafting rate increases, with superior cell proliferation for grafted groups compared with nongrafted groups and, even more important, a greater number of cells on grafted 2 when confronted with the less grafted group. The results confirm previous findings on the relation of grafting degree and cell proliferation [28] and reaffirm that measuring GD by TB assay can be a strong tool for anticipating surface behavior after modification treatments.

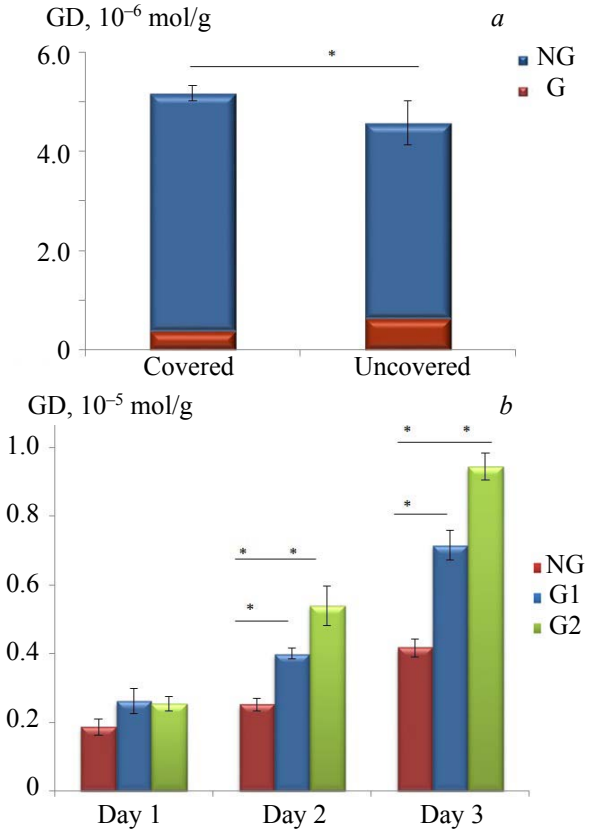

Fig. 4. a) GD of PCL samples measured in experiments protected or not from light exposure. b) Primary sheep fibroblast cell growth over different pNaSS grafting degrees. PCL samples for 1, 3, and 7 days of culture.

Conclusions. This note investigates the influence of the definition of the parameters on the quantification of grafted groups using the toluidine blue O colorimetric assay. All pH values of the staining, staining time, decomplexation time, and light exposure conditions have shown a significant influence on the final measurement of the grafting degree, as follows. Acid staining (pH 5) led to insufficient complexation and consequently lower grafting measurement. On the other hand, staining pH over 10 induced nonspecific absorption, which can be translated by an overestimation of the number of grafted groups. The originally proposed 5-h staining was too short to complete the saturation of PCL surfaces. At least 6 h was necessary to complete the decomplexation of the toluidine blue O. The decomplexation process for PCL samples took about 24 h. After this point, no increment was verified (up to 72 h of decomplexation). The results confirm toluidine blue O photosensitivity, and to ensure optimal quantification the assay should be conducted away from the light. Finally, FTIR analysis indicates that nonspecific interaction has very little influence on the measurements against specific absorption, and cell culture assay results showed superior cell proliferation over samples with higher grafting density measured by the toluidine blue O technique. The combination of these results implies that the assay, if conducted carefully, is a reliable method for identifying surface composition and a good indicator of the surface behavior after grafting treatment.

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