Accuracy of the dimethylmethylene blue spectrophotometric assay in measuring the amount of encapsulated pentosan polysulfate into nanoparticles

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OBJECTIVES

Pentosan polysulfate (PPS), a highly sulfated semisynthetic polysaccharide, chemically and structurally resembles glycosaminoglycans (GAGs). Therefore, it was used here the dimethylmethylene blue (DMMB) binding assay, which is a rapid spectrophotometric assay that is widely used to measure sulfated GAGs (1, 2), to estimate the amount of PPS encapsulated into chitosan-based nanoparticles (NPs).

EXPERIMENTAL METHODS

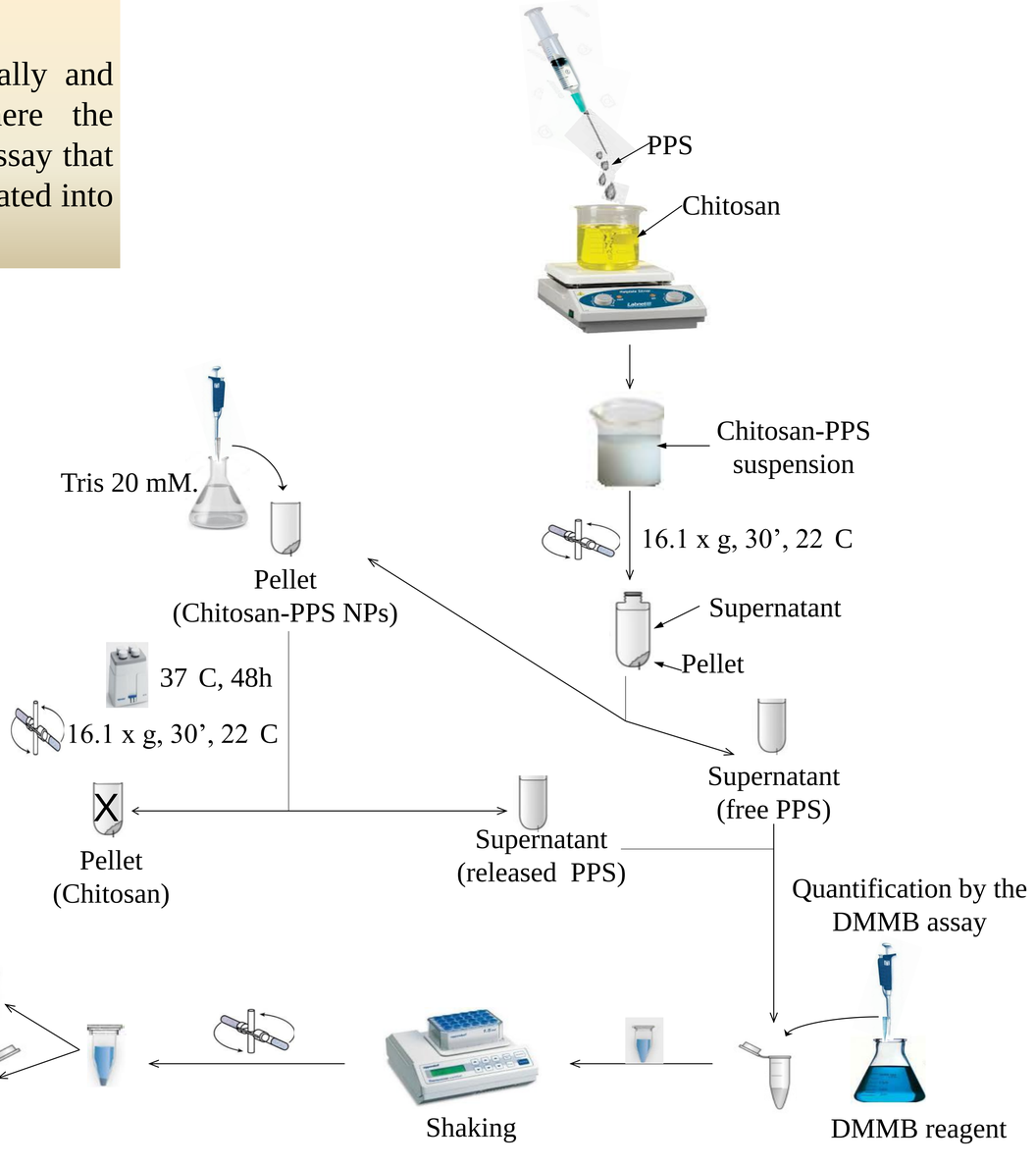


Figure 1-.Schematic configuration of the experimental procedures used for the preparation of the NPs and the determination of PPS entrapment efficiency..

- Test NPs were prepared by drop-wise addition of PPS (1 mg/ml) to chitosan (1 mg/ml) under continuous magnetic stirring. The NPs were separated by centrifugation.
- The encapsulated PPS into NPs was quantified either by measuring the free PPS (indirect method) or by direct measurement of the entrapped PPS after releasing from NPs by incubation in Tris buffer followed by centrifugation. Drug entrapment efficiency of the test NPs was calculated from the ratio between the amount of encapsulated PPS into NPs and the total amount of PPS added x 100.
- PPS was measured by the DMMB assay (2).

RESULTS AND DISCUSSION

PPS was detected by the DMMB dye in a linear relationship at both low and high concentrations, as shown in **Figure 2**.

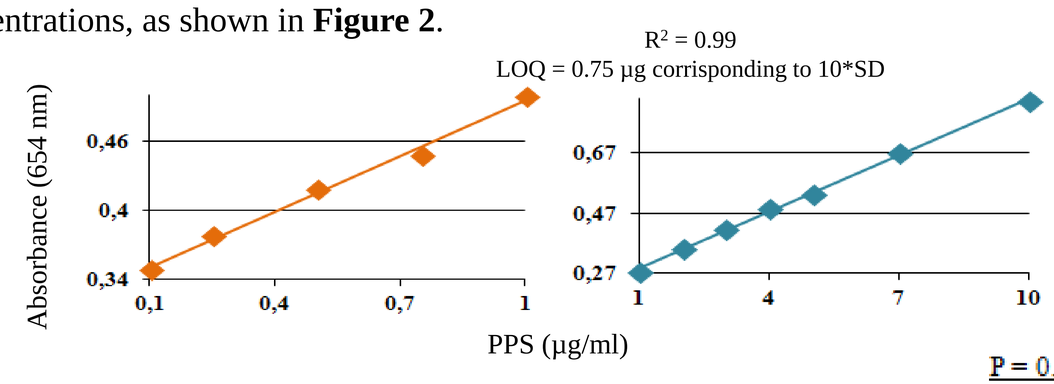
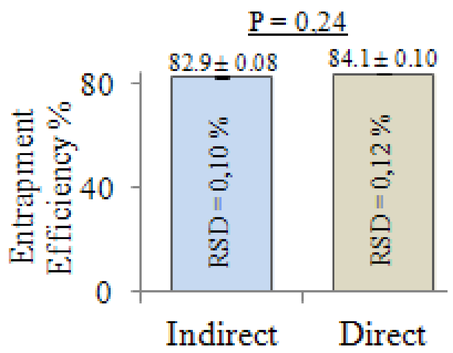


Figure 3 shows NPs entrapment efficiency estimation (direct and indirect) and the relative measurement uncertainty.
The measurement uncertainty expressed by the relative standard deviation (RSD) suggests that the DMMB assay is highly reliable in measuring the PPS entrapped amount.



These results are collectively in agreement with previously published data obtained by the capillary zone electrophoresis (CZE) (3), as shown in **Table 2**. However, all the uncertainties related to results produced by the DMMB assay are much smaller (at least 30-fold) than those produced by the CZE, indicating its higher accuracy over other detection assays.

Table 2. Comparison between the DMMB assay and CZE in measuring PPS and determining its loading efficiency into chitosan-based NPs

Detection method	%Entrapment efficiency (Indirect)	%Entrapment efficiency (Direct)	LOQ (mg/ml)	Linearity	%DFA[(b)]	Accuracy error	%RSD[(a)]	%Recovery
DMMB	82.9 ± 0.084	84.1 ± 0.10	≤ 0.00075	R = 0.998	2.0 – 2.86	0.004 – 0.02	0.10 – 3.27	97.14 - 98
CZE	82.4 ± 2.5	84.7 ± 4.8	< 0.27	R = 0.986	5.0 – 8.57	0.01 – 0.06	4.69 – 10.0	91.4 - 95

DFA: difference from actual; RSD: relative standard deviation; LOQ: quantification limit; CZE: capillary zone electrophoresis; NPs: nanoparticles. Values are average SD of at least 3 independent assays performed in duplicate. (a) Acceptance limit of precision: %RSD ≤ 15%. (b) Acceptance limit of accuracy: %DFA < 15%.

CONCLUSIONS

The DMMB assay demonstrated to be able to estimate the PPS entrapment efficiency into the NPs with high accuracy both within and between the assay. Moreover, the DMMB dye showed high stability and binding specificity reflected by the absence of any interaction with nonsulfated molecules present in a mixture with the PPS.

After spiking of 0.2, 0.4 and 0.7 mg of PPS with chitosan solution (1 mg/ml), the DMMB assay measured quantities of PPS that were very close to those nominal, as shown in **Figure 4**. This result indicates the high accuracy and precision of the DMMB assay (**Table 1)**.

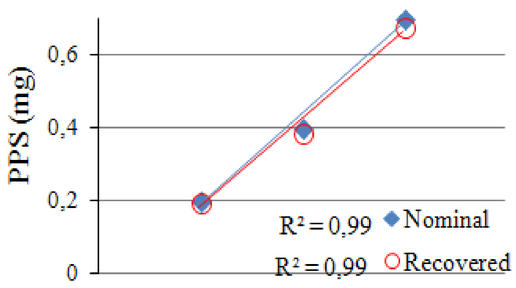
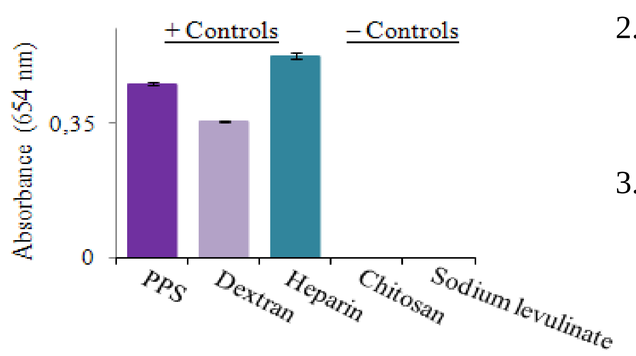


Table 1. Accuracy and precision of the DMMB assay in measuring spiked PPS

Spiked PPS (mgs)	Recovered PPS (mgs)	%RSD[(a)]	%DFA[(b)]
0.2	0.196 ± 0.006	3.27	2.00
0.4	0.39 ± 0.01	2.56	2.50
0.7	0.68 ± 0.0007	0.10	2.86

DFA: difference from actual; RSD: relative standard deviation; Values are average SD of at least 3 independent assays performed in duplicate. (a) Acceptance limit of precision: %RSD ≤ 15%. (b) Acceptance limit of accuracy: %DFA < 15%.

Sodium levulinate (the unique excipient in the PPS solution) and chitosan neither interacted with the DMMB dye nor interfered with the measurement of the PPS, as shown in **Figure 5**. GAGs-positive controls reacted with the DMMB but differently both in quantitative and qualitative terms, with respect to the PPS and to each other (**Figure 5**).



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3. Abdel-Haq H, Bossù E. Capillary electrophoresis as a tool for the characterization of pentosan nanoparticles. J Chromatogr A. 2012; 1257:125-30.