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Application Note

Performance of Estapor® Microspheres in a Latex Turbidimetry Immunoassay for C-reactive Protein (CRP)

Introduction

Latex Turbidimetry ImmunoAssay (LTIA) has been determined as a homogeneous assay technique that allows for quick quantification of a specific biomarker at low concentrations[1]. It is one of the most common tests used for human sample testing in clinical chemistry laboratories due to full automation in random access platforms, as well as its high throughput[2].

The sensitivity of turbidimetric immunoassays can be increased using microparticles as the solid phase of the assay (particle-enhanced turbidimetric immunoassay). In a typical format, antibodies are linked to latex particles / microspheres (LPs), which enhances light scattering as the latex particles aggregate due to biomarker binding by the antibody.

Selection of latex particles is crucial for the development of any LTIA. Particle size and surface chemistry are among the most important parameters to consider. Typically, particle sizes for LTIA range from 100 nm to 300 nm, but in some assays smaller particles of ~50nm and larger particles up to 800 nm have been successfully used[3]. The selection will depend on the desired analytical performance and the optical requirements of a particular system.

Most particles/microspheres for LTIA applications are made of polystyrene. Plain or non-functionalized particles are used for protein adsorption, in which the protein binds the particle surface by hydrophobic and ionic interactions. For this type of particle, proteins can adhere in multiple layers onto the surface, their spatial orientation being somewhat variable and non-specific. Alternatively, particles can also be surface modified thus allowing covalent coupling to the ligand. Carboxyl and primary amine groups are the most common modifications. Covalent conjugation is more specific and less variable than protein adsorption thereby improving the sensitivity and stability of the reagent, yielding a colloidally stable system with the required surface concentration of immobilized proteins[3].

For surface-modified latex particles the surface charge density or the amount of functional groups on its surface expressed as µeq/g is another parameter to be considered. It can also be expressed as Parking Area ($Å^2$/group), the area between each functional group, and is dependent on the particle density, diameter and surface charge density.

This technical note explores the effect of particle size and surface chemistry on the analytical performance of four types of latex particles/microspheres from Estapor®, plain (Plain-LPs) and carboxyl-modified (COOH-LPs) particles, with two different sizes for each. For this purpose, C-reactive Protein (CRP), a well-known biomarker used in many immunoassays related with infections, chronic inflammatory and cardiovascular diseases[4], is used as the clinical model. This protein shows a wide clinical range of interest that makes it a good tool to assess the effect of particle characteristics on analytical parameters such as sensitivity, dynamic range and the 'Hook' or 'Prozone' effect. Typically, CRP full detection range is up to 150 mg/L, while a high sensitivity detection range includes low CRP concentrations ranging from 0.1 to 5 mg/L.

Materials and Methods

Key Raw Materials:

- Monoclonal antibody Anti-human CRP was coated on the particles.
- Bovine Serum Albumin (BSA, Ref. 81-003-3, Millipore®) was used to block the surface of the particles after antibody coupling.
- N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC, Ref. 341006, Merck) and N-Hydroxysuccinimide (NHS, Ref. 130672, Merck) were used to activate COOH groups on latex particles before coupling the antibody.
- CE-marked CRP Latex Turbidimetry Reagent was used as reference method for correlation method studies.

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Table 1: Estapor® LTIA Microspheres

Product Name	Cat No.	Lot No.	Mean Diameter Specification (nm)	Mean Diameter of lot used in this study (nm)	COOH surface content Specification (µeq/g)	COOH surface content of lot used in this study (µeq/g)
K 010	39469081	M4616	81 - 125	100	N/A	N/A
K 030	23690087	3412	276 - 325	281	N/A	N/A
PSI 90-21	80380012	3365	80 – 150	101	100 – 200	169 (Parking Area: 55)
IT 250	80380626	M4220	220 - 275	256	40 – 100	58 (Parking Area: 63)

- 7 serum controls from BIO-RAD, with concentration ranging from 0.78 to 189 mg/L of CRP (determined by Beckman Coulter platform) are used for assay optimisation.
- All data presented in this technical note was obtained by testing 40 human serum samples with CRP concentration distributed along the full range from 0.5 to 111 mg/L.

Assay Workflow:

Monoclonal antibody was coated either by adsorption or covalently onto LPs (prior activation of COOH groups was completed where appropriate). During all washing steps, LPs were centrifuged and resuspended in next step buffer. Main conjugation steps are detailed as follows and summarised in **Table 2**.

1. Both COOH and Plain-LPs were first washed to remove any additive present from particle synthesis
2. An activation step was performed for COOH-LPs by addition of EDAC and NHS to activate carboxyl groups through carbodiimide chemistry.
3. Antibody coupling was carried out for 2 hours for COOH -LPs and 4 hours for Plain-LPs at room temperature in a pH 8 coupling buffer. After centrifugation, the supernatant was retained to determine coupling efficiency by BCA protein quantification assay.
4. A blocking step was performed overnight at room temperature.
5. All LPs were resuspended and stored at 2-8°C.

A standard turbidimetric method was applied in the Clinical Chemistry Analyser, where Reagent 1 (R1) is first added, followed by human serum sample addition and Reagent 2 (R2) addition. R1 contains assay buffer (100 mM HEPES, 154 mM NaCl, 0.5% BSA, 0.03% ProClin™ 300, pH 7.5) and R2 conjugated latex particles (100 mM HEPES, 0.2% BSA, 0.03% ProClin™ 300, pH 7.5). After R2 addition, agglutination kinetics measurement was performed at 578 nm for all particles. Note: selecting a lower wavelength would have increased sensitivity and benefit smaller size particles, as higher optical contrast would have been achieved and lower particle concentration would have been needed. However, 578 nm wavelength was selected aiming to stay as close as possible to commercial immunoturbidimetric assays, the majority of which run at higher wavelengths in order to avoid matrix interferences (e.g. lipids, haemoglobin or bilirubin). In order to compensate for this effect the particle concentration on R2 was adjusted as indicated in **Table 3** in order to achieve similar background for each particle type (about 1.0 AU) thus allowing comparison.

Table 3: Summary of Particle Concentration in R2

COOH-LPs		Plain-LPs	
101 nm	256 nm	100 nm	281 nm
0.30%	0.06%	0.30%	0.04%

Table 2: Summary of Conjugation Conditions

Step	Buffer	COOH-LPs		Plain-LPs	
		101 nm	256 nm	100 nm	281 nm
1) Washing	Buffer	25 mM MES, pH 6.1		25 mM MES, pH 6.1	
	Centrifugation	100 min 17000 rcf	15 min 17000 rcf	100 min 17000 rcf	15 min 17000 rcf
2) Activation	Buffer	25 mM MES, pH 6.1 1 COOH:5 EDAC/NHS (molar:molar)		Not required	
3) Antibody Coupling	Buffer	50 mM HEPES, pH= 8.0		50 mM HEPES, pH= 8.0	
	µg IgG/mg LP	70	28	212	76
	Incubation	2 hours @room temperature		4 hours @room temperature	
4) Blocking	Buffer	50 mM HEPES, 1% BSA, pH= 8.0		50 mM HEPES, 1% BSA, pH= 8.0	
	Incubation	Overnight @room temperature		Overnight @room temperature	
5) Storage	Buffer	100 mM HEPES, 0.2% BSA, 0.03% ProClin™ 300, pH 7.5		100 mM HEPES, 0.2% BSA, 0.03% ProClin™ 300, pH 7.5	

Results and Discussion

The effect of particle size and surface chemistry on analytical performance for four types of latex particles from Estapor®, plain and carboxyl-modified particles, with two different sizes for each is discussed. All particles were coupled to the same monoclonal antibody against CRP following an optimised protocol for each particle in order to get the highest response of each particle reference. Parameters such as surface saturation, coupling pH, and incubation times were considered to maximise the response of each particle. Plain-LPs required at least three times more antibody to achieve the same response level as COOH-LPs; however, the conjugation protocol is easier for Plain-LPs as they do not require activation of carboxylic groups before antibody coupling, thus needing fewer centrifugation and dispersion steps. The smaller (diameter: 100 nm) LPs show slower agglutination kinetics thus longer reading times are required to maximise the response. The optimal response was achieved within an interval of 491 sec, while for larger (diameter: 250/280 nm) LPs a longer reading time promotes prozone effect, while a shorter reading time (205 sec) helps to reduce the prozone while maintaining sensitivity.

The effect of surface chemistry (COOH & Plain) and particle size (100 & 250/280nm) is discussed next.

Impact of Particle Surface chemistry (COOH & Plain)

The effect of two different surface chemistries, carboxylated particles (COOH-LPs) and plain particles (Plain-LPs) is evaluated for the two ranges of interest, full range detection (from 1 to 100 mg/L CRP) using small particles of 100 nm and low range detection (from 0.1 to 5 mg/L) using larger particles of 250/280 nm.

Figure 1 shows Passing-Bablok regression for both, COOH-LPs (left) and Plain-LPs (right) in the full range detection (100 nm particles). Both surface chemistries studied showed good correlation with a CE-marked LTIA reagent, confidence interval comprises slope of 1 for both particles. However, none of the particles include an intercept value of zero in their confidence interval and Plain-LPs gave slightly higher results than COOH-LPs. This is due to additional reagent optimisation being required to achieve reagent equivalence, which we believe is achievable, primarily from an analytical sensitivity point of view. Pearsons correlation coefficient was found to be 0.995 for both surface chemistry particles confirming that both chemistries are equally good options to be used in full range detection.

To obtain this data, Plain-LPs needed three times additional antibody amount to achieve the same response level as COOH-LPs. It is demonstrated that both Plain-LPs and COOH-LPs can be optimised for the detection of biomarkers in wide-range detection with similar analytical response.

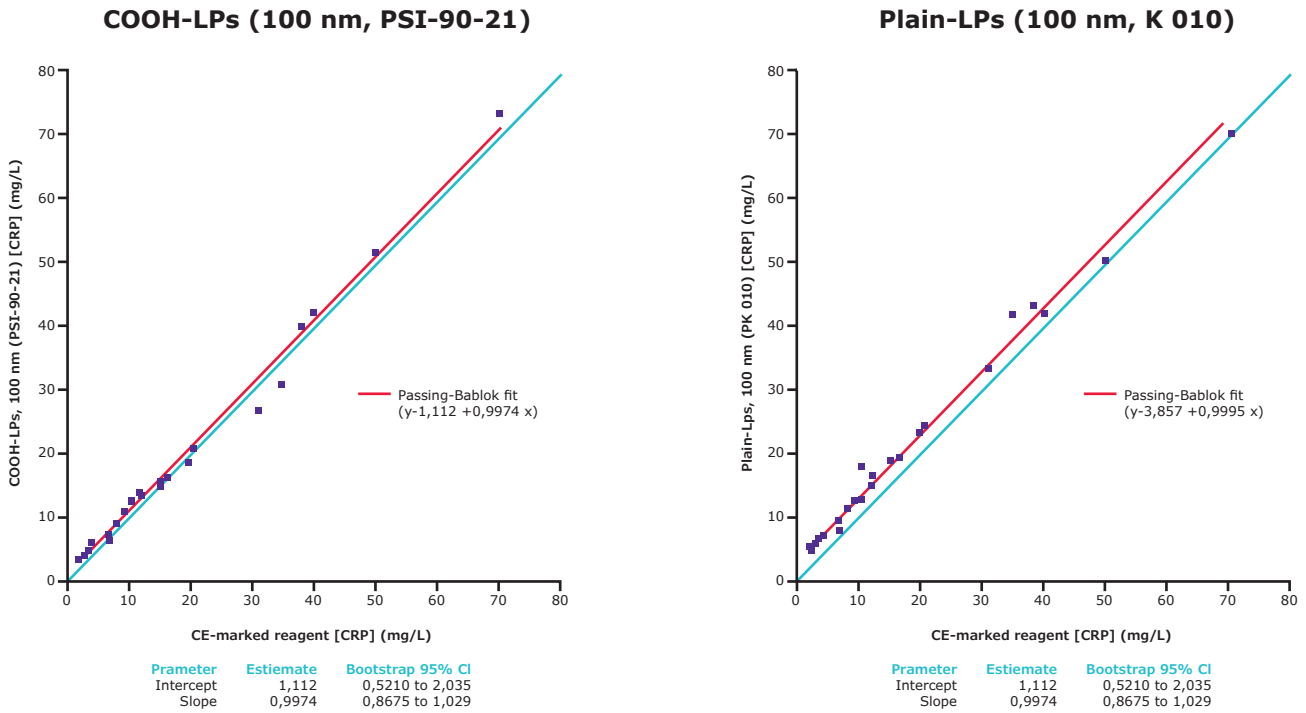

Figure 1 Passing-Bablok regression analysis for COOH-LPs (left) and Plain-LPs (right) for the full range detection using 100 nm particles. 24 human serum samples tested in duplicate. Red line: fitted regression line. Blue line: Identity. The equation and 95% confidence intervals for the intercept and slope for the regression line are shown in each panel.

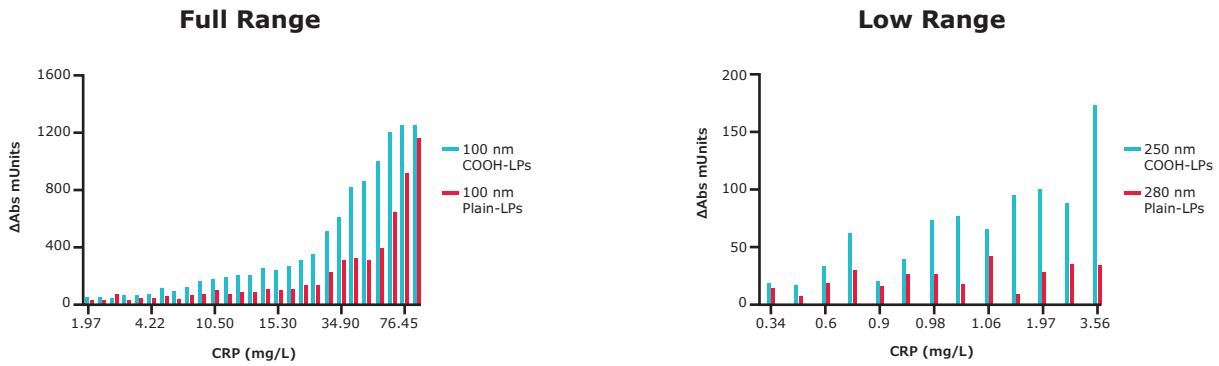

Figure 2 Comparison of COOH-LPs (blue bars) and Plain-LPs (red bars) for both full detection range (left) up to 100 mg/L (smaller particles 100 nm size) and high sensitivity detection range (right) up to 4 mg/L (larger particles 250/280 nm size). Each human serum samples tested in duplicate.

Figure 2 shows the data obtained by testing human serum samples for the full range (left) and the low range (right) using both COOH-LPs and Plain-LPs. In all samples, higher absorbance unit values were achieved for COOH-LPs (black bars), likely due to the better antibody orientation on the particle surface. A method correlation study was not carried out for the larger particles as Plain-LPs would require additional modifications such as the addition of an agglutination enhancer to the assay buffer (R1) to be functional in the high sensitivity detection range. On the contrary, the response was sufficient to achieve good correlation with the CE-marked reagent for the full range detection, as discussed previously (**Figure 1**).

Impact of Particle Size (100 & 250/280 nm)

The effect of particle size is evaluated for both surface chemistries under study (COOH & Plain). **Figure 3** (left) shows the analytical response obtained for COOH-LPs at both particle sizes, 100 nm (filled circle) and 250 nm (empty circle). It is observed how full range detection is only achieved for small particles while large particles showed prozone effect after 20 mg/L (**Figure 3**, left). On the contrary, greater absorbance values are showed by larger particles in the high sensitivity range up to 4 mg/L CRP (**Figure 3**, right). This effect is due to the agglutination kinetics of each particle size, it is well-known that larger particles have increased probability of collision and thus they achieve faster agglutination, whereas smaller particle size and their slow agglutination kinetics facilitated the detection on the wide range without showing saturation or prozone effects.

Figure 4 shows the results obtained for Plain-LPs at both particle size 100 nm (filled circle) and 280 nm (empty circle) in the high sensitivity range. For COOH-LPs, greater response in observed for larger particles for the low range, thus for both surface chemistries same performance is demonstrated. Faster agglutination of 280 nm LPs facilitates determination of CRP concentrations in the high sensitivity range.

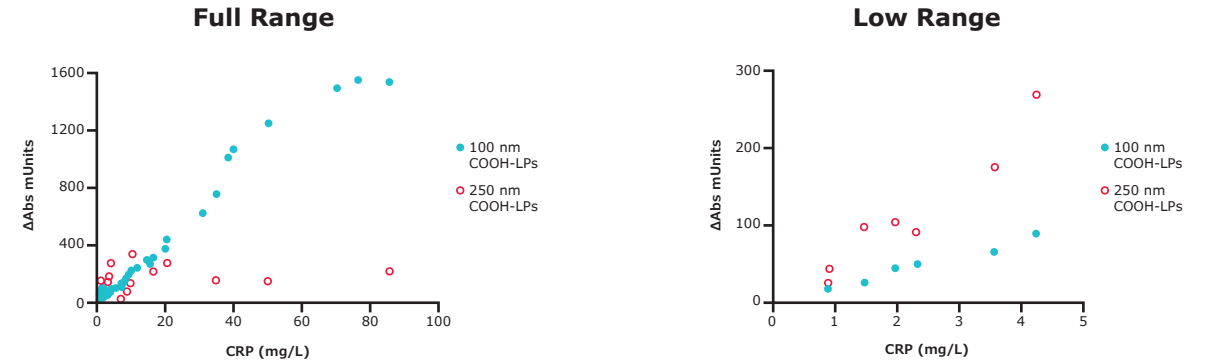

Figure 3 Comparison of 100 nm (blue dots) and 250 nm (red dots) particle size for COOH-LPs in the full detection range (left) and high sensitivity range (right). Each human serum samples tested in duplicate.

Significantly lower response is observed when comparing Plain-LPs (**Figure 4**) and COOH-LPs (**Figure 3**, right). It is demonstrated than Plain-LPs require not only higher amounts of antibody coupled but also the addition of an agglutination enhancer in the assay buffer (R1) to be functional in the high sensitivity detection range.

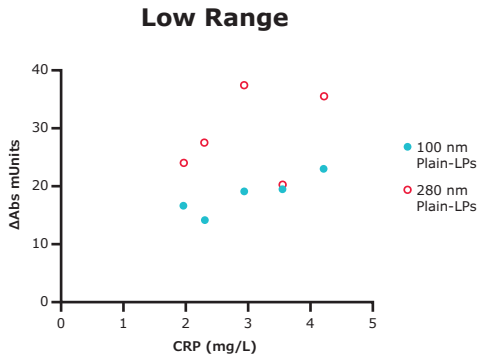

Figure 4 Comparison of 100 nm (blue dots) and 280 nm (red dots) particle size for Plain-LPs in the high sensitivity range. 5 human serum samples tested in duplicate.

Conclusions

This technical note discussed the effect of surface chemistry and microsphere particle size on performance of a common latex-enhanced immunoturbidimetry assay for CRP.

It has been demonstrated that a wide range immunoturbidimetric reagent could be developed using both surface chemistries under study, carboxylated and plain. No meaningful differences have been observed between chemistries for the full range detection up to 100 mg/L of CRP using 100 nm particles. Even though Plain-LPs required at least 3 times more antibody to achieve similar performance than COOH-LPs, they can be a good particle for the development of wide-range systems because of the simplicity of their conjugation process which avoids carboxylic group activation steps. On the contrary, a clear decrease in response is demonstrated for low concentration range detection when using larger Plain-LPs. The higher response obtained thanks to the antibody orientation provided by the COOH-LPs needs to be compensated by the addition of agglutination enhancers in the assay buffer when using Plain-LPs in the low range detection using larger particles.

Particle size is one of the most important parameters to consider when selecting microspheres for a particular system. This technical note demonstrates the effect of small particles (100 nm) and larger particles (250/280 nm). Detection on the full range up to 100 mg/L of CRP is only achievable for small particles (100 nm). Larger particles show prozone effect at 20 mg/L of CRP and increased response on the low range up to 5 mg/L of CRP. Both chemistries studied, COOH and Plain, showed the same particle size effect, however Plain LPs will require further optimisation to achieve good sensitivity in the low range.

In conclusion, both 100 nm particles, Plain-LPs (K 010) and COOH-LPs (K 010 / K 030 / PSI 90-21), showed similar detection capability in the full range up to 100 mg/L. However, in the low range, larger particles are required, Plain-LPs (K 030) or COOH-LPs (IT 250), being the COOH-LPs showing an increased response. Plain-LPs require additional optimisation (increased antibody amount or addition of agglutination enhancers) to achieve same performance than COOH-LPs for systems demanding high sensitivity.

References:

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