Modification of the Stewart biphasic colorimetric assay for stable and accurate quantitative determination of Pluronic and Tetronic block copolymers for application in biological systems

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Abstract

Block copolymers are increasingly being applied in areas such as transfection, membrane sealing, site-specific targeting, and bionano-engineering yet there is a relative paucity of assays available for simple, stable and reproducible colorimetric determination of copolymer concentration in solution. We have focused on improving the accuracy and reproducibility of a modified version of the Stewart biphasic colorimetric assay for quantitative determination of Pluronic (poloxamer) and Tetronic (poloxamine) macromolecules. The optimized assay achieved linear response ranges in chloroform for commonly used copolymers such as poloxamine 904 (20–300 µg/ml), poloxamine 908 (10–400 µg/ml), poloxamer 402 (20–400 µg/ml), and poloxamer 407 (10–400 µg/ml). Variation in the type of chlorinated solvent used significantly increased assay sensitivity, presumably through macromolecular reorientation, affording increased access for copolymer–ferrothiocyanate complexation. This was found to be optimally favored by the planar geometry of the solvent cis 1,2-dichloroethylene. For application to biological systems copolymer–protein interactions were for the first time determined and were found to be dependent on the fraction of hydrophobic constituents of the block copolymers and protein type. For instance serum albumin was found to interact with copolymers of low hydrophilic–lipophilic balance values and poly(propylene oxide) contaminants, whereas this interaction was not significant with the relatively hydrophilic IgG. In such systems the colorimetric assay directly determines the fraction of unbound (free) copolymer in the presence of proteins.

Keywords: Poloxamer; Poloxamine; Block copolymer; Polyethylene oxide; Ammonium ferroisothiocyanate; Chlorinated solvent; Copolymer–protein interaction

Pluronic (poloxamers) and Tetronic (poloxamines) block copolymers are increasingly finding diverse applications in membrane biochemistry, transfection, and immunization protocols and in nanobioengineering and nanomedicine [1–4]. Although highly desirable, at present there is no simple, stable, rapid, and reliable quantitative assay procedure available to determine the concentration of these macromolecules in solution.

To our knowledge five colorimetric methods have been applied to the assay of certain Pluronics. These include the cobalt thiocyanate method [5–9], the Wickbold method [10], the potassium tetrakis (4-halophenyl) borate method [11], an iodine-based assay [12,13], and the modified ammonium ferrothiocyanate method [14], which is based on Stewart’s classical phospholipid assay [15]. The first three procedures were either unsuitable in their methodology for applications which require small sample handling, had the disadvantage of requiring complex equipment, or were too time consuming. Alternatively, the iodine-based assay [12,13], which is suitable for low-volume handling, is unstable,
and generates erratic absorbance values, and is therefore analytically imprecise.

The biphatic colorimetric assay described by Stewart [15] for phospholipids was recently modified for small-volume handling and quantitative determination of Pluronics by Govender et al. [14]. This determination is based on complex formation between the ethylene oxide constituents of the copolymer and the ammonium ferroisothiocyanate reagent. However, the ammonium ferroisothiocyanate reagent is known to decompose in aqueous solution to give Fe\(^{2+}\) and thiocyanogen, with the latter eventually hydrolyzing to sulfate [16,17]. The reduction potentials of these species in the organic solvents used to extract the complex are not known but it is likely that similar reactions could occur [14], leading to time-related decrease in absorbance value that can generate erroneous readings and underestimation of copolymer concentration. This is of significant importance with respect to analysis of low concentrations of block copolymers in bionanoengineering and nanomedicine [1,4]. Thus the aim of this work was to resolve the current instability of the modified Stewart assay [14] to ensure accurate quantitative determination of Pluronic and Tetronic copolymers in solution.

**Experimental**

**Materials**

All poloxamers and poloxamines were the kind gift of BASF Aktiengesellschaft (Ludwigshafen, Germany) and these were used as received. Flocor was the kind gift from Dr. J. M. Grindel, CytRx Corp. (GA, USA). Analar-grade KCl was supplied by Fisher Scientific (UK). Analar-grade anhydrous ferric chloride, ammonium thiocyanate, chloroform, and analogues were purchased from Sigma Aldrich (UK) as was cis\textit{trans} 1,2-dichloroethylene 98%. Ammonium ferroisothiocyanate solution contained ammonium thiocyanate (0.4 M) and anhydrous ferric chloride (0.1 M) in distilled water. Bovine serum albumin (BSA)\(^1\) and rat IgG were supplied by Sigma (UK).

**Copolymer characterization**

The equivalent poly(ethyleneglycol) (PEG)/poly(ethylene oxide) (PEO) average molecular weights of poloxamers and poloxamines were determined by the method of raised temperature gel permeation chromatography as described in detail previously [18]. This is essential due to batch-to-batch molecular weight variation (polydispersity) frequently found in commercial products [4] and potential variation due to for example in-process degradation. The ethylene oxide content of copolymers was determined by \(^1\)H NMR spectroscopy (Bruker WM 360 MHz) in deuterio-

**Abbreviations used:** BSA, bovine serum albumin; PEG, poly(ethyleneglycol); PEO, poly(ethylene oxide); AA, atomic absorptionmetry; ICP-AES, inductively coupled plasma–atomic emission spectroscopy.

**Colorimetric assay procedure**

The assay was carried out in Eppendorf tubes (1.5 ml) containing 0.5 ml of an appropriate chlorinated solvent (dichloromethane, 1,2-dichloroethane, 1,1-dichloroethene, cis\textit{trans} 1,2-dichloroethylene and 1,3-dichloropropane), 0.5 ml ammonium ferroisothiocyanate solution, and 0.5 ml phosphate buffer (pH 7.4) containing the required quantity of poloxamer or poloxamine. The system was shaken vigorously for 20 min, followed by centrifugation (3 min) using a microfuge at 16,000 \(g\) at room temperature (22 °C). The lower chlorinated solvent layer was extracted and placed into a quartz cuvette with a 1-cm pathlength and then covered with 100 µl of the ammonium ferroisothiocyanate reagent. The absorbance was measured at 510 nm in a Helios \(\beta\) Unicam Spectrophotometer (Spectronic Unicam-UK). Incubations were performed in triplicate and each experiment was repeated at least three times.

**Equilibration time**

To determine the shaking time needed to ensure that equilibrium in the biphatic system is reached the assay was performed with 200 \(\mu\)g of the selected poloxamer or poloxamine that was shaken over a range of different time periods (1–20 min). Following centrifugation at room temperature (3 min, 16,000 \(g\)) the absorbance of the organic phase was determined at 510 nm as described above.

**Determination of complex stability**

Measurement of complex stability was carried out using a Cary 50 Scan UV–Visible Spectrophotometer (Varian-UK) set to scan from 200 to 800 nm over time. The scanning test was carried out with the analyte contained in a quartz cuvette either in the absence or in the presence of a surface layer of ammonium ferroisothiocyanate solution (100 µl).

**Iron content determination**

Ammonium ferroisothiocyanate solution (5 ml) was mixed with an appropriate amount of copolymer dissolved in water (5 ml), followed by addition of chloroform or cis\textit{trans} 1,2-dichloroethylene (5 ml). The mixture was shaken for 20 min and centrifuged at room temperature (16,000 \(g\), 10 min). For atomic absorptiometry (AA) a sample of the organic layer (2 ml) was removed and transferred to a small tube. EDTA solution (5 ml, 0.05 M) was added and the mixture was shaken for 5 min. The sample then underwent centrifugation (ibid) and the aqueous layer was
assayed for Fe determination using a Perkin–Elmer (Germany) 1100B Model flame atomic absorption spectrophotometer, equipped with an Fe lamp using a wavelength of 248.3 nm and lamp current of 40 mA. The instrument was calibrated using standard Fe solutions (0.5–3 ppm Fe) prepared from commercial concentrates. Iron content was also determined by inductively coupled plasma–atomic emission spectrometry (ICP-AES). Here, 1 ml of the organic layer was evaporated to dryness on a hotplate, and the residue was dissolved in concentrated HNO₃ and made up to 5 ml, giving a final acid concentration of 5% v/v HNO₃. Initially, calibration curves were constructed with standard solutions of Fe in the range of 0 to 1 ppm using the emission at 238.204 nm.

**Results and discussion**

**Assessment of complex stability**

Once the organic layer was removed from the biphasic system for spectrophotometric analysis we noted that the absorbance readings were decreasing with time (Fig. 1), which had not been reported previously [14]. For example in the absence of the covering aqueous reagent (monophase system) the absorbance of the complex in chloroform decreased by approximately 10% within 1 h and by 80% after 10 h (Fig. 1A). In the presence of the covering aqueous reagent layer the complex was stable with a loss of not more than 0.5% in absorbance after 12 h (Fig. 1B). This pattern of complex instability in the absence of the ammonium ferroisothiocyanate reagent was observed for all poloxamers and poloxamines tested in a range of chlorinated solvents (representative examples are shown in Figs. 1C and D) and was in accord with the earlier observations of ammonium ferroisothiocyanate decomposition in aqueous solution [16,17]. Neither the addition of ammonium thiocyanate nor the solutions of oxidizing agents halted the degradation of the complex and the loss of thiocyanate peaks at 2100 and 800 cm⁻¹ in the IR spectra was confirmed (data not shown). Therefore, the aqueous reagent layer appears to act as a reservoir of Fe³⁺ thiocyanate and maintains the equilibrium [16,17] following loss of Fe³⁺ from the complex in the organic phase. This highlights the absolute necessity for rapid assay of

![Fig. 1. Spectrophotometric indication of poloxamine 908–ferroisothiocyanate complex stability in organic solvent–air (A and C) and solvent–reagent (B and D) interfaces. Copolymer concentration was 200 μg/ml in all cases.](image-url)
the organic layer especially when dealing with low polymer concentrations.

**Equilibration time**

On average the shaking time required in both chlorinated solvents (chloroform and 1,2-dichloroethylene) to attain an equilibrium state between the copolymer and the complex was 5 min (data not shown) despite variation in absorbance sensitivity in both solvents. To ensure complete copolymer/complex equilibration prior to colorimetric assay we chose 20 min as the set shaking time in all cases. These observations bring into question the validity of shaking times previously used below 2.5 min with chloroform as solvent [14], which could result in underestimation of copolymer concentration.

**Solvent effect**

The type of chlorinated solvent used affects the sensitivity of the assay with an increased absorbance observable at equivalent copolymer concentration (poloxamine 908, 200 µg/ml), thus suggesting increased access of the ammonium ferroisothiocyanate for complex formation (Fig. 2). Of the solvents initially tested 1,1-dichloroethene had the lowest sensitivity with cis/trans 1,2-dichloroethylene being the most sensitive; each of 1,3-dichloropropane, 1,2-dichloroethane, dichloromethane, and cis/trans 1,2-dichloroethylene were significantly (1.8×, 2.2×, 2.4×, and 2.5×, respectively) more sensitive than chloroform. The variable sensitivity may be due to solvent ordering of the gross macromolecular structure, resulting in enhanced access of reagent for complex formation. Such a dimensional ordering phenomenon, in the case of poloxamers, has been reported previously with polar solvents, partially insoluble polar solvents [19], and nonchlorinated organic solvents [20]. It appears that the complex interacts primarily with the ethylene oxide portion of the macromolecules since assay using poly(propylene oxide) (Mn = 3500, viscosity at 25°C = 1300 cps) resulted in insignificant molar absorptivity irrespective of solvent type compared with block copolymers (data not shown). Our preliminary investigation has further confirmed that these industrially manufactured block copolymers are optically inactive at the concentration range used (up to 400 µg/ml) which was also well below their respective critical reverse micelle concentrations in chlorinated solvents. These observations suggest that the variation in absorption is most likely a solvent effect. However, future experiments with small-angle X-ray scattering and circular dichroism may reveal the underlying mechanism(s).

To further elaborate on the solvent effect the solvent structure–activity relationships show that the optimal complex formation is favored in the ethene series by the presence of cis chlorine atoms with planar geometry because sensitivity decreases with analogues, 1,3-chloro groups, or single carbon molecules. To test this notion next we assessed cis and trans 1,2-dichloroethylene individually and in contrast to the racemic mixture the pure cis isomer was found to instill markedly increased sensitivity with an absorbance of 1.13 following a two-fold dilution at the start of the assay. The trans isomer did not show any significant absorbance (data not shown), confirming that optimal geometry for complex formation in the series tested is the cis 1,2-dichloroethylene. The trends observed in the methane series complex formation are consistent because absorption is again optimized by the presence of two chlorine atoms. Chloroform and dichloromethane have been reported as suitable solvents for PEO extraction due to their hydrogen-bond-donating capability [21] and it is likely that this interaction is fundamental to our observations with the different solvents. These results confirm that the assay can be applied effectively with a wide range of chlorinated solvents affording the flexibility of different solvation systems at highly applicable sensitivities. From these results we decided that for comparative purposes we would use chloroform and cis/trans 1,2-dichloroethylene because these afforded an optimal assay range without the require-

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**Fig. 2.** Effects of various chlorinated solvents on poloxamine 908–ferroisothiocyanate complex formation. Copolymer concentration was 200 µg/ml in all cases.
ment of a dilution step that was necessary with the pure cis 1,2-dichloroethylene.

**Determination of Fe content**

Both AA and ICP-AES afforded very similar results for Fe content (Table 1) for the copolymers tested in chloroform and cis/trans 1,2-dichloroethylene. As expected in both solvent systems there is an increase in Fe content with increase in molecular weight of copolymer. This would further support a difference in solvent-induced copolymer reorientation in each of the two solvents tested. The greater association of Fe in the 1,2-dichloroethylene would indicate that the copolymers are solvated in such a fashion that they are more linear (open chain) in the case of the chloroalkene, thus allowing greater opportunity for complex formation with the ethylene oxide groups along their length compared to the chloroform.

<table>
<thead>
<tr>
<th>Copolymer</th>
<th>Mol % PEO</th>
<th>PEG/PEO Equivalent Mw</th>
<th>Polydispersity index</th>
<th>µmol Fe/µmol copolymer AA Chloroform</th>
<th>µmol Fe/µmol copolymer 1,2-Dichloroethylene</th>
<th>µmol Fe/µmol copolymer ICP-AES Chloroform</th>
<th>µmol Fe/µmol copolymer ICP-AES 1,2-Dichloroethylene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poloxamine 908</td>
<td>77.8</td>
<td>22455</td>
<td>1.69</td>
<td>7.99 ± 0.56</td>
<td>21.7 ± 0.82</td>
<td>7.18 ± 0.2</td>
<td>24.04 ± 3.42</td>
</tr>
<tr>
<td>Poloxamine 904</td>
<td>37.9</td>
<td>3193</td>
<td>1.46</td>
<td>0.99 ± 0.07</td>
<td>2.35 ± 0.05</td>
<td>1.05 ± 0.05</td>
<td>3.03 ± 0.09</td>
</tr>
<tr>
<td>Poloxamine 901</td>
<td>8.2</td>
<td>2070</td>
<td>1.18</td>
<td>0.59 ± 0.07</td>
<td>0.91 ± 0.10</td>
<td>0.69 ± 0.02</td>
<td>1.29 ± 0.11</td>
</tr>
<tr>
<td>Poloxamer 407</td>
<td>71.2</td>
<td>11930</td>
<td>1.31</td>
<td>3.15 ± 0.60</td>
<td>11.24 ± 0.68</td>
<td>3.35 ± 0.85</td>
<td>10.48 ± 0.54</td>
</tr>
<tr>
<td>Poloxamer 402</td>
<td>18.5</td>
<td>2210</td>
<td>1.34</td>
<td>0.70 ± 0.02</td>
<td>1.96 ± 0.13</td>
<td>0.60 ± 0.07</td>
<td>2.02 ± 0.16</td>
</tr>
<tr>
<td>Poloxamer 188</td>
<td>83.2</td>
<td>7417</td>
<td>1.27</td>
<td>1.86 ± 0.23</td>
<td>10.64 ± 0.39</td>
<td>1.79 ± 0.18</td>
<td>11.28 ± 0.63</td>
</tr>
<tr>
<td>Flocor</td>
<td>78.1</td>
<td>8753</td>
<td>1.04</td>
<td>Not determined</td>
<td>Not determined</td>
<td>Not determined</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

Fig. 3. Calibration plots for poloxamines (A and B) and poloxamers (C and D) in chloroform (●) and 1,2-dichloroethylene (○). Error bars were within 5% of mean value and for clarity have been omitted.
Calibration plots

The linear response ranges for selected poloxamers and poloxamines varied depending on the copolymer in use and the organic solvent (i.e., chloroform or cis/trans 1,2-dichloroethylene). In all cases a greater linear range was achieved with chloroform, cf. cis/trans 1,2-dichloroethylene (Fig. 3). Goodness of fit ($R^2$) for all calibration plots was 0.9961 or better, showing excellent correlation with small intercepts (Fig. 3). These sensitivity ranges are directly applicable to nanomedicine and bionanoengineering applications [1–4].

Influence of proteins

The biphasic assay has been applied previously [14] to the quantitative determination of Pluronics directly in biological fluids. However, potential discrepancies could arise from the acidic nature of the reagent, which could cause protein aggregation leading to false results, and from copolymer–protein interaction. With regard to the latter it has been shown that poloxamer 188 can interact with plasma components such as serum albumin [22], complement proteins, and various lipoproteins [18]. In addition direct poloxamine 908–protein interaction at lattice surfaces has been observed in real time using surface plasmon resonance [23]. Therefore to address copolymer–protein interaction we based our assay on serum albumin as an example of the most abundant plasma protein and IgG, which is a relatively hydrophilic protein. Selected copolymers (poloxamine 908 and poloxamer 402) were incubated with varying concentrations of protein (0–200 µg/ml) after which the protein components were precipitated prior to colorimetric analysis. Following incubation with albumin (25–200 µg/ml), poloxamine 908 absorbance values initially declined rapidly (from 0.31 to 0.24 units) but then stabilized to give near-constant absorbance values (Fig. 4A). In the case of poloxamer 402, which has a far lower hydrophilic–lipophilic balance value than poloxamine 908, absorbance values reduced continuously with increasing protein concentration (0–200 µg/ml) (Fig. 4A). This indicated that the albumin was interacting favorably with the more hydrophobic poloxamer 402 and suggested that the significant loss of absorbance with poloxamine 908 may be due to a hydrophobic component (POP and or components with high POP content) of this polydisperse (PDI = 1.69) copolymer. To test this, we then assayed poloxamine 908 in the presence of IgG (Fig. 4A). This resulted in reduced absorption loss (0.02 units) compared to the greater reduction in the presence of albumin (0.06 units). Unfortunately there are no pure (i.e., containing a defined fraction) preparations of poloxamine 908 or poloxamer 402 available commercially. To corroborate this hypothesis we repeated the experiment with both poloxamer 188 (PDI = 1.27) and a near-monodisperse pure preparation, Flocor (PDI = 1.04). Interestingly we found that the poloxamer 188 absorbance reduced with increasing albumin concentration yet the Flocor showed only slight reduction in absorbance followed by remarkably constant absorbance readings with increasing albumin concentration (25–200 µg/ml) (Fig. 4B). This further supported the hypothesis that a hydrophobic...
population within the polydisperse poloxamer 188 preparation is being taken up by the albumin. Further evidence for the hydrophobic constituent removal came from a double incubation experiment (Fig. 4C). Here, poloxamine 908 loss was determined following an initial incubation with albumin (100 μg/ml). The supernatant, following drying and reconstitution with water, was then reincubated with albumin. Again, following protein precipitation and supernatant assay for poloxamine, no further polymer loss was detected, confirming that a minor hydrophobic population of macromolecules is being taken up by the albumin. In light of this observation the colorimetric assay is essentially determining the fraction of unbound (free) copolymers.

Conclusions

This is the first demonstration of a stable, simple, and versatile colorimetric assay for the determination of poloxamers and poloxamines in solution. The sensitivity of the assay can be adjusted by the use of various chlorinated organic solvents, which appear to control orientation of the macromolecules, thus facilitating ferroisothiocyanate complex formation presumably through hydrogen bonding. The relative stability, simplicity, and defined biological applicability of this assay provides an important bioanalytical tool, which is highly applicable for quantitative determination of ethylene-oxide-containing macromolecules, thus facilitating ferroisothiocyanate complex formation presumably through hydrogen bonding.

References