

Self-assembled polymer vesicles in deciding action of Zn-sulfanilamide allergenicity

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Abstract Allergic reactions to sulfonamide-based drugs are quite common; hence, medications containing sulfonamides are prescribed carefully. Metal ion complexation may block the nitrogen binding site of sulfonamide by complexation and reduce such responses. In this study, trace concentrations of Zn were found to bind with sulfanilamide at pH~1. The complexation was studied in water as well as in vesicular medium of polyethylene glycol (PEG) and a block copolymer, PPG-PEG-PPG. The binding constants (k) of Zn-sulfanilamide complex were determined in water, PEG, and PPG-PEG-PPG block copolymer media. The values suggest that complexation occurs best in water followed by PPG-PEG-PPG, PEG #4000, and PEG #12000. Though the binding constants are high in water and block copolymer media, the complex is not very stable as the absorbance value for the complex was found to decline with time. The same complex when prepared in polymer matrix shows higher stability. The results prompted us to explore the extraction possibilities of the Zn-sulfanilamide complex by using aqueous biphasic extraction systems comprising the polymers against sodium sulfate solution. The complex was analyzed for its allergenic response in different media by competitive enzyme-linked immunosorbent assay (ELISA) technique. The allergic response of the compounds in the respective media is the

resultant of the binding constant and the stability of the complex in that particular medium.

Keywords Sulfanilamide · Polyethylene glycol · Block copolymer · ELISA · Allergenicity

Introduction

Sulfonamides are a group of synthetic drugs used to combat the infection caused by gram-positive and gram-negative bacteria, fungi, and certain protozoans [1]. With the advent of antibiotics, the use of sulfadugs has declined, but still, they occupy an important place among antibacterial pharmaceuticals [2, 3]. The drug has got special importance after the rampage of HIV by its ability to protect and treat *Pneumocystis jirovecii* infections so common in AIDS. Recently, the synthesis of metal sulfanilamide compounds has received much attention due to their enhanced antibacterial action and enhanced lipophilicity. Complexation of metal ion with sulfanilamide increases solubility and permeability of barriers to get into cells; this in turn increases bioavailability and hence the activity [4]. Furthermore, sulfadugs and their metal complexes possess many applications as diuretic, antiglaucoma, or antiepileptic drugs, among others [5–10]. Several reports on the antimicrobial activity of sulfanilamides and their metal complexes are also available [11, 12]. Studies on the metal compounds have much physiological and pharmacological relevance because the metal chelates of sulfadugs have been found to be more bacteriostatic than the drugs themselves [13].

About 3 % of patients receiving sulfonamide antibiotics experience true hypersensitivity reactions [14]. Appearance of mild rashes is the most common symptom. These generally occur within 1 week of drug therapy and disappear after 7 to 14 days of discontinuation [15, 16]. Serious dermatologic

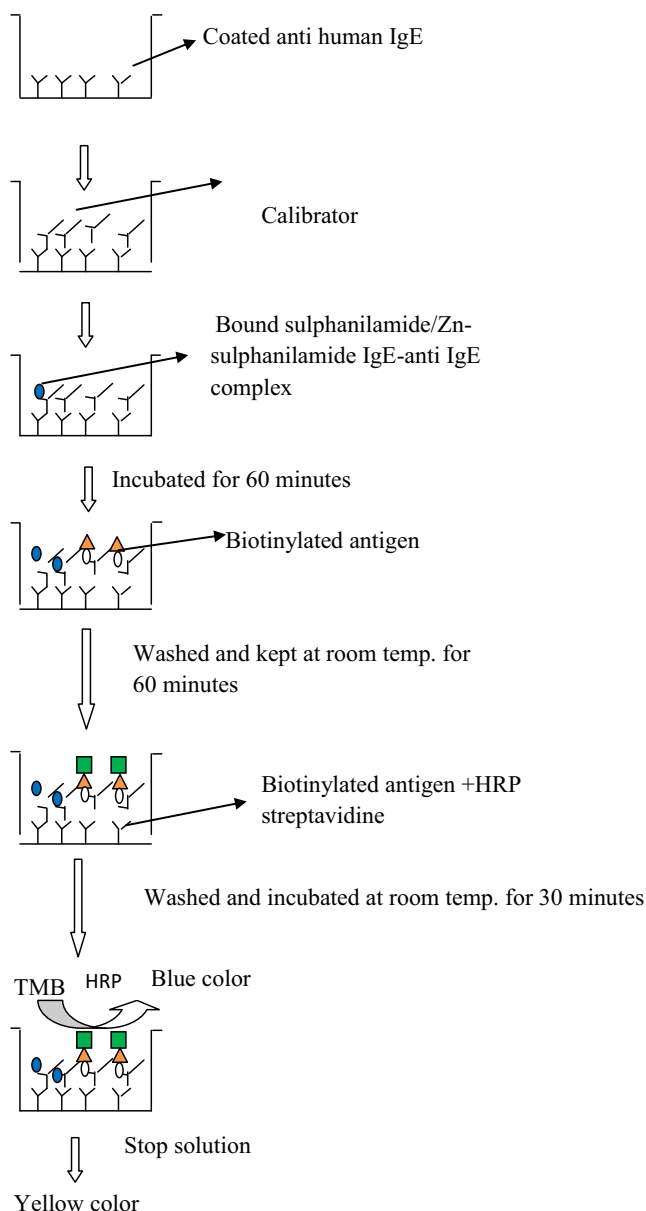
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reactions such as Stevens-Johnson syndrome and toxic epidermal necrosis are, however, less frequent. Non-dermatological reactions such as drug fever, serum sickness syndrome, vasculitis, nephritis, hepatitis, and hemolytic anemia have also been reported [15]. Metal complexation of drug molecules may significantly alter the antibody binding and hence reduce the allergenicity.

Recent developments on polymer therapeutics is one of the most challenging and promising trends in biomedical technology [17, 18]. Polymer drug carriers are being used with a special focus on site-specific delivery and controlled release of drug molecules to the diseased cells [19]. Once the drug molecules enter the polymeric vesicles, they have altered stability, and this property



Scheme 1 Experimental details of the competitive ELISA test

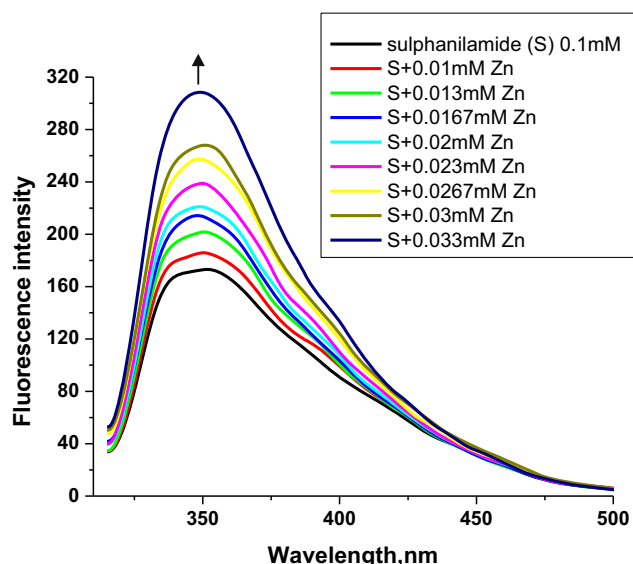


Fig. 1 Fluorescence emission intensities of sulfanilamide with increasing concentrations of Zn

may be exploited to have better possibilities of treatment. During drug processing and characterization, separation of a particular drug molecule is required from certain media. Aqueous biphasic extractions have long been used to separate and purify several drug molecules from their aqueous solutions [20, 21]. Drug molecules may get extracted in the micellar medium of a polymer from an aqueous solution by using an environmentally benign technology.

The present article reports on the complexation of sulfanilamide with Zn in water as well as in vesicular medium of polyethylene glycol (PEG) and a block copolymer, PPG-PEG-PPG. The binding constants (k) of Zn-sulfanilamide complex in water, PEG, and PPG-PEG-PPG block copolymer media suggest that complexation occurs best in water followed by PPG-PEG-PPG, PEG #4000, and PEG #12000. The complex was analyzed for allergenic response in these media by competitive enzyme-linked immunosorbent assay (ELISA) technique.

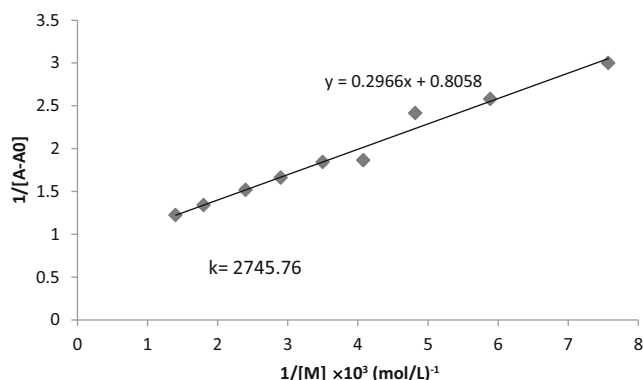


Fig. 2 Binding constant of Zn-sulfanilamide complex in water

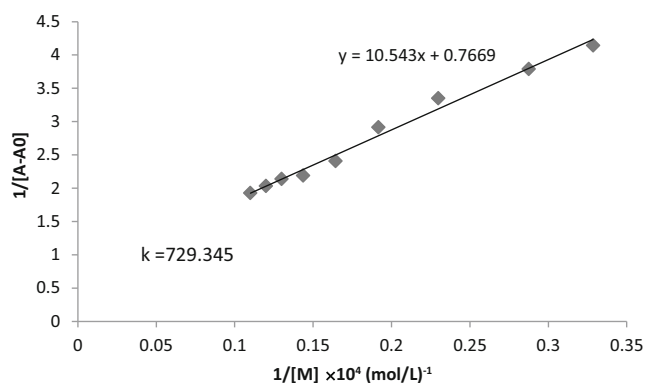


Fig. 3 Binding constant of Zn-sulfanilamide complex in PEG #4000

Experimental

Materials Polyethylene glycol (#4000, #12000) (Merck), polypropylene glycol-polyethylene glycol-polypropylene glycol block copolymer (PPG-PEG-PPG, #2000) (Aldrich), sulfanilamide (Indian Drug & Pharmaceuticals LTD.), zinc sulfate, 1-(2-pyridylazo)-2-naphthol (PAN), sodium sulfate (G.R., min. 99.5 %), and all other chemicals were of analytical grade and used as received. Allerbind Master Allergy Kit (48 tests) was purchased from Lilac Medicare (P) LTD for ELISA.

Apparatus The UV visible spectra were obtained using U-3501 Hitachi spectrophotometer. Mettler Toledo seven compact pH/Ion meter S220 was used to measure and adjust the pH of different solutions. A luminescence spectrometer LS-55B (Perkin-Elmer, USA) was used for monitoring fluorescence property. LISSCAN II (Erba) was used to compare the allergenicity by comparing the absorbance at 450 nm.

Preparation of reagents Two-millimolar solutions of zinc sulfate and sulfanilamide were prepared by weighing; 50 % (w/v) PEG #12000, 50 % (w/v) PEG #4000, and 60 % (w/v) PPG-PEG-PPG were prepared by dissolving appropriate amounts in triple-distilled water.

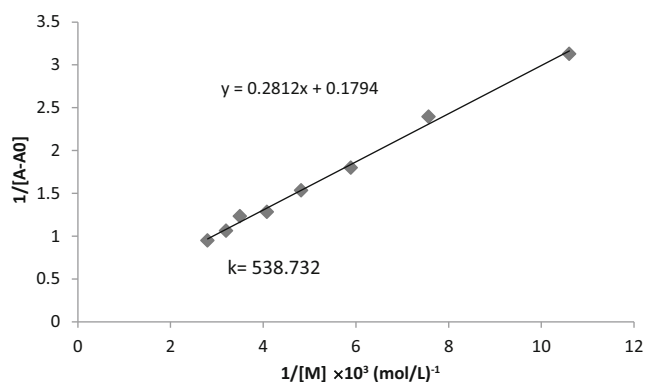


Fig. 4 Binding constant of Zn-sulfanilamide complex in PEG #12000

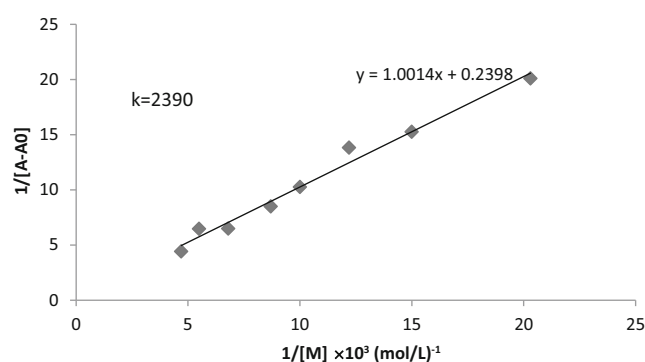


Fig. 5 Binding constant of Zn-sulfanilamide complex in PPG-PEG-PPG block copolymer

Study of binding constant Increasing volumes of 1 mM solution of zinc were added to 1 mL of 1 mM sulfanilamide, in 0.1 M HCl/KCl buffer (pH 1) in the respective media, and the absorbance data were taken for each set of complexation.

The binding constant values of the Zn-sulfanilamide complex were studied in water, PEG #4000, PEG #12000, and PPG-PEG-PPG block copolymer media by using the Benesi-Hildebrand (BH) equations involving the absorbance data of the complexes [22].

$$\frac{1}{A - A_0} = \frac{1}{A_1} + \frac{1}{(A_1 - A_0)K[M]} \quad (1)$$

$$Y = A + BX \quad (2)$$

where

- A is the absorbance of the experimental solution containing Zn and sulfanilamide
- A_0 is the absorbance of the sulfanilamide only
- A_1 is the absorbance when Zn is completely bound with sulfanilamide
- $[M]$ is the metal ion concentration
- K is the binding/association constant

In order to find the fate of the complexes in different media, the absorbance time scans in respective media were also recorded.

Aqueous biphasic extraction For aqueous biphasic extraction of Zn(II), the ABS was prepared by mixing 3-mL solutions of the above PEG #4000 or PEG #12000 or PPG-PEG-PPG block copolymer and 3 mL Na₂SO₄ (2 M) in 15-mL graduated

Table 1 Binding constant (k) values of Zn-sulfanilamide complex in different media

Medium	k (at 25 °C) (mol ⁻¹ L)
Water	2745.76±120.25
PPG-PEG-PPG	2390.00±118.32
PEG #4000	729.34±36.45
PEG #12000	538.73±26.90

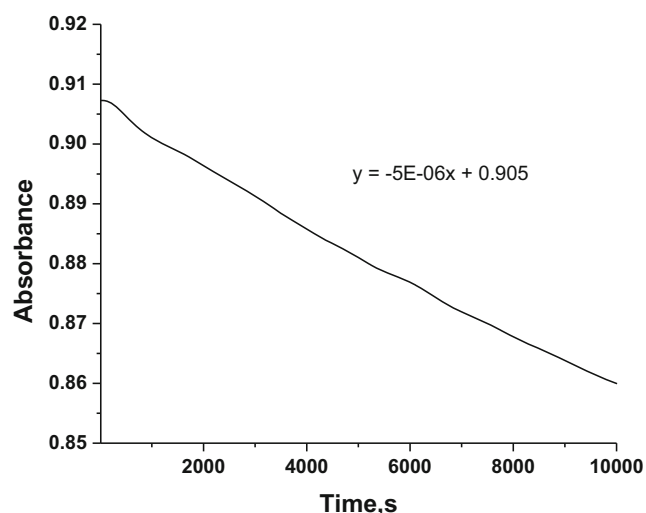


Fig. 6 Decay of absorbance of Zn-sulfanilamide complex in water with time

test tubes. This salt was found to form an equal volume phase separation with the proposed polymer solutions. The Zn-sulfanilamide complex was prepared in pH 1 buffer medium and added to the aqueous biphasic systems formed from PEG #4000/ Na_2SO_4 , PEG #12000/ Na_2SO_4 , and PPG-PEG-PPG/ Na_2SO_4 solutions at different pH values of the salt-rich phase. After 10 min each of shaking and centrifugation, the polymer-rich phase from the top was carefully withdrawn and analyzed for Zn using 1-(2-pyridylazo)-2-naphthol (PAN) as a complexing agent in pH 4.8 acetate buffer medium. The results were compared with a suitable blank containing sulfanilamide only in the absence of Zn.

ELISA test to analyze the allergenicity of sulfanilamide and Zn-sulfanilamide complex The ELISA has been used as a diagnostic tool in medicine to detect and compare the allergic

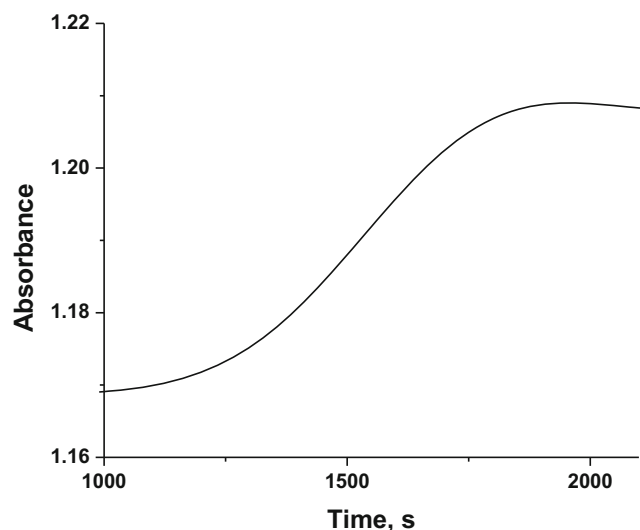


Fig. 7 Growth of absorbance of Zn-sulfanilamide complex in PEG #4000 with time

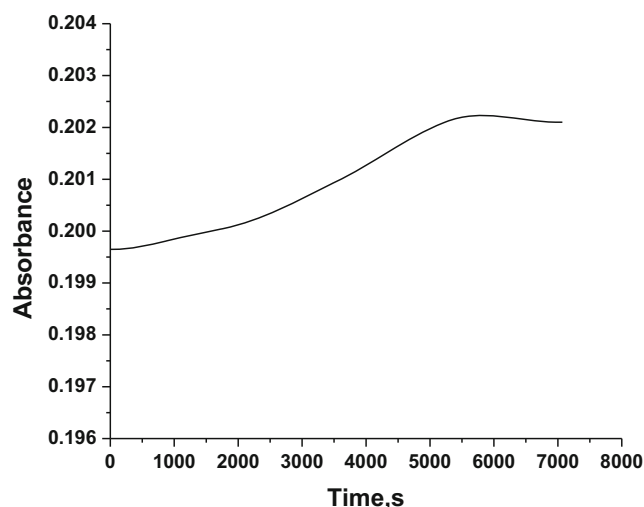


Fig. 8 Growth of absorbance of Zn-sulfanilamide complex in PEG #12000 with time

response of several substances. The ELISA assay starts with antihuman IgE-coated microwells; 25 μL of calibrators containing different concentrations of IgE was added to these microwells, and 25 μL of sulfanilamide/Zn-sulfanilamide complex in different media was also added. The plates were covered and incubated for 60 min at room temperature. During this time, two events occur: (i) binding of IgE in calibrator to antihuman IgE in the wells and (ii) binding of sulfanilamide/Zn-sulfanilamide (allergen) to the bound IgE of calibrator according to allergenicity of the complex.

The plates were washed thrice with 500 μL of wash buffer every time to remove the unbound reactants; 100 μL of biotinylated allergen (allergen provided in the kit) was next added and kept covered at room temperature for 1 h. The biotinylated allergen reacted with the unbound arm of the antibody which did not bind sulfanilamide/Zn-sulfanilamide

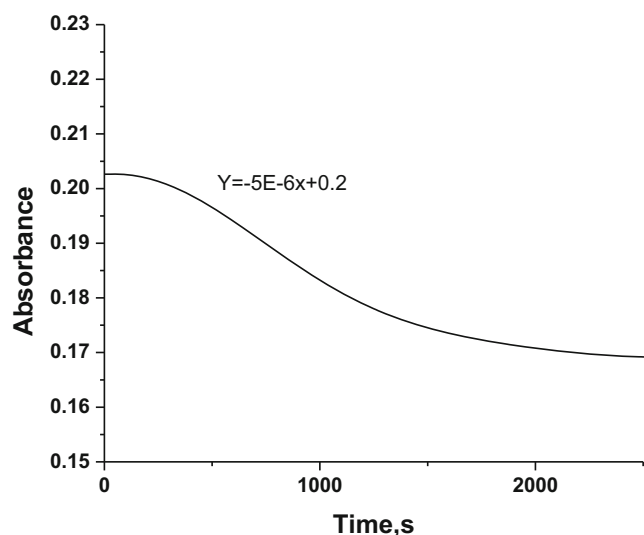


Fig. 9 Decay of absorbance of Zn-sulfanilamide complex in PPG-PEG-PEG block copolymer with time

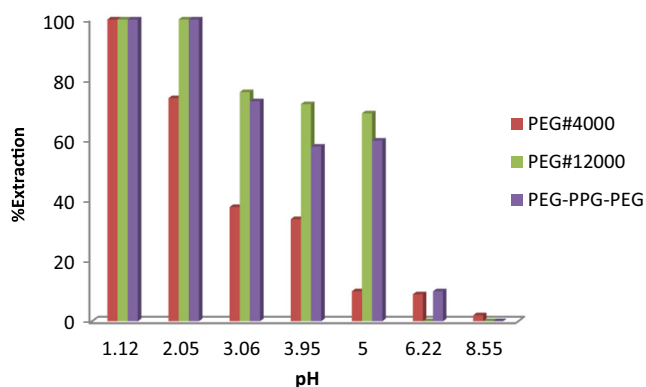


Fig. 10 Extraction profile of Zn-sulfanilamide complex in different polymer media from sodium sulfate salt-rich phase

complex in the previous reaction. Thus, more biotinylated allergens will bind to the wells if a lesser amount of sulfanilamide/Zn-sulfanilamide complex had bound in the previous step. As binding signifies allergenicity, a lesser amount of Zn-sulfanilamide binding means a lesser allergic compound.

The plates were washed again as before; 100 μ L of streptavidin horseradish peroxidase (HRP) conjugate was added next. Streptavidine binds to biotin and binds the HRP to the well. The plates were covered and incubated at room temperature for 30 min. The plates were once again washed, and 100 μ L of 3,3',5,5'-tetramethylbenzidine (TMB) was added following which the plates were covered and incubated at dark for 30 min at room temperature. TMB formed a blue color in proportion to the biotinylated allergen binding; 100 μ L of stop solution (acid) was added next, and absorbance was taken at 450 nm after 5 min. Addition of stop solution yielded a yellow color in proportion to the blue color (Scheme 1).

Results and discussion

According to Pearson's hard and soft acids and bases (HSAB) theory, sulfur atoms prefer binding with soft metal ions like Cu^+ , Ag^+ , Hg^{2+} , Cd^{2+} , etc. [23]. In addition to this, the presence of sulfur along with nitrogen atoms in sulfanilamide

enhances the coordination propensity of the ligand to bind the borderline metal ion Zn^{2+} . It is envisaged that a complex formation between the sulfanilamide and Zn^{2+} might increase the fluorescence intensity of the system via suppression of photoinduced electron transfer (PET) from the electron-rich π -bonded sulfur atoms to the phenyl unit. Both sulfanilamide and its metal complex exhibit an absorption band around 296 nm which is assigned to the phenyl π - π^* transition. The complexation is associated with enhanced fluorescence signals with increasing metal ion concentration (Fig. 1).

The complex shows increasing absorbance values with increasing metal ion concentration, and the results are taken for calculating the binding constant values following Eq. 1. The binding constants are calculated from absorbance measurements in all the four media, viz. water (Fig. 2), 50 % PEG #4000 (Fig. 3), 50 % PEG #12000 (Fig. 4), and 60 % PPG-PEG-PPG block copolymer (Fig. 5) solutions. Results (Table 1) indicate that water medium offers the highest binding constant value followed by the block copolymer, PEG #4000, and PEG #12000. This is as per the expectations because PEG molecules self-assemble in water to form vesicles [24]. Complexation is facilitated in pure water, having no vesicular interference as in the PEGs. The block copolymer offers lower hindrance in complexation as the alternating PEG-PPG-PEG blocks do not allow the system to self-assemble as in PEG, allowing a higher value of binding constant for the complex.

The complexes in their respective media were analyzed for their time-dependent stability by using their absorption time scan. It was observed that in water medium, the complex was unstable and breaks down with a decay rate of 5×10^{-6} absorbance units per second (Fig. 6). However, in the PEG media (both PEG #4000 and PEG #12000), the absorbance slowly increases with time, indicating the formation of the complex in vesicular media followed by stabilization. Figures 7 and 8 indicate that the time to stabilize the complex is lower (30 min) for the lower molecular weight PEG #4000 and higher (1 h) for the higher molecular weight PEG #12000, having a longer chain length. In the block copolymer medium, the complex behaves more or less as in water medium, as it can be seen from Fig. 9 that the decay rate is almost the same as that in water medium (5×10^{-6} absorbance units per second). However, it is also seen that the complex stabilizes after

Table 2 Absorbance values obtained from the ELISA test

Sample	Absorbance 1 (0.35 IU/mL)	Absorbance 2 (0.71 IU/mL)	Absorbance 3 (3.51 IU/mL)
2 mM sulfanilamide in water medium	0.079	0.088	0.178
2 mM zinc-sulfanilamide in water medium	0.087	0.154	0.189
Zinc-sulfanilamide complex in PEG #4000	0.102	0.103	0.124
Zinc-sulfanilamide complex in PEG #12000	0.082	0.090	0.120
Zinc-sulfanilamide complex in PPG-PEG-PPG	0.071	0.077	0.113

half an hour and there is no further decrease in the absorbance value. This is probably due to hydrogen bonding stabilization offered by the polymeric glycol moiety of the block copolymer.

Illuminated by the results of complexation in the polymeric medium, we tried to see the possibility of aqueous biphasic extractions of Zn as Zn-sulfanilamide complex from different pH values of the salt-rich phase. Figure 10 shows the extraction profile of Zn-sulfanilamide complex in different polymer media from sodium sulfate salt-rich phase. As the complex is most suitably formed at pH 1, it was found that the extractions from pH 1 of the salt-rich phase are most suitable in all the three polymeric solutions studied. As zinc is one of the important essential trace elements in many biological systems, its extraction as a sulfanilamide complex in the polymer-rich phase of aqueous biphasic system may be of immense application.

The results of the competitive ELISA test of the compounds in different media are tabulated in Table 2. Since a higher absorbance value indicates lower allergenic response (as discussed in the “Experimental” section), it is evident that the Zn-sulfanilamide complex is lesser allergenic than sulfanilamide alone in water medium. The allergenicity further decreases if the complex is taken in the vesicular medium of PEG #4000. With low concentrations of antibody (0.35 and 0.71 IU/mL), significant increase in absorbance was noted when the complex is taken in PEG #4000 medium, signifying better antiallergic compounds. The observed allergic response of the compounds in their respective media was found to be a resultant of the binding constant and the stability of the complex in that particular medium. The allergenicity is higher in PEG #12000 and block copolymer which may either be due to lower binding constant of the complex or due to declining stability of the complex in the block copolymer medium.

Conclusion

The complexation of sulfanilamide with Zn has been studied in different polymeric media in addition to pure aqueous solution at pH 1. Absorbance and fluorescence spectral studies confirm the complexation. Binding constants of the complex in different media calculated from the absorbance data indicate that the binding is highest in water medium followed by the block copolymer, PEG #4000, and PEG #12000. Absorbance time scan of the complex in these media indicates that the complex is less stable in pure water and in block copolymer media. Due to vesicular medium in PEG, the complex slowly grows and then attains a stable configuration in both PEG #4000 and PEG #12000. The complexation is useful in aqueous biphasic extraction of Zn from pH 1 of the salt-rich phase. Finally, the allergenicity of the complex has been compared in different media directly with sulfanilamide.

The results are in favor of decreased allergenicity of the complex than sulfanilamide itself, and the effect is most pronounced in PEG #4000 medium.

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Conflict of interest There is no conflict of interest regarding the article. No animal studies were performed.

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