Original Article

Preliminary Assessment of Various Additives on the Specific Reactivity of Anti- rHBsAg Monoclonal Antibodies

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Received: 6 May 2015
Accepted: 12 Jul 2015

Abstract

Background: Antibodies have a wide application in diagnosis and treatment. In order to maintain optimal stability of various functional parts of antibodies such as antigen binding sites, several approaches have been suggested. Using additives such as polysaccharides and polyols is one of the main methods in protecting antibodies against aggregation or degradation in the formulation. The aim of this study was to evaluate the protective effect of various additives on the specific reactivity of monoclonal antibodies (mAbs) against recombinant HBsAg (rHBsAg) epitopes.

Methods: To estimate the protective effect of different additives on the stability of antibody against conformational epitopes (S3 antibody) and linear epitopes (S7 and S11 antibodies) of rHBsAg, heat shock at 37 *°C* was performed in liquid and solid phases. Environmental factors were considered to be constant. The specific reactivity of antibodies was evaluated using ELISA method. The data were analyzed using SPSS software by Mann-Whitney nonparametric test with the confidence interval of 95%.

Results: Our results showed that 0.25 *M* sucrose, 0.04 *M* trehalose and 0.5% BSA had the most protective effect on maintaining the reactivity of mAbs (S3) against conformational epitopes of rHBsAg. Results obtained from S7 and S11 mAbs against linear characteristics showed minor differences. The most efficient protective additives were 0.04 *M* trehalose and 1 *M* sucrose.

Conclusion: Nowadays, application of appropriate additives is important for increasing the stability of antibodies. It was concluded that sucrose, trehalose and BSA have considerable effects on the specific reactivity of anti rHBsAg mAbs during long storage.

Avicenna J Med Biotech 2015; 7(4): 145-150

Keywords: Epitopes, Monoclonal antibodies (mAbs), Polysaccharides

Introduction

Monoclonal antibodies have extensive applications for diagnosis and therapeutic purposes [1,2]. The quality of mAbs depends on the molecular structure, which relies on the reaction conditions, storage and confounding factors in the stability of antibody [3]. Increasing the efficiency and biological half-life of antibodies has been a major challenge for scientific centers and trading companies [4]. The biological half-life of the products refers to the amount of time that they can demonstrate 90% of their initial performance [5]. In a previous study carried out based on monoclonal and polyclonal antibodies, a sensitive homemade ELISA kit was developed for detection of hepatitis B surface antigen [6]. Produced antibodies had reactivity in two different epitopes; antibodies recognizing conformational epitopes and antibodies recognizing linear epitopes [7]. The obtained results indicated that homemade assay had high sensitivity and specificity in comparison to commercial kits in detecting HBsAg in biological and standard samples. Some studies showed that the half-life of the designed kit was not acceptable as a diagnostic kit [6]. Modifying environmental factors and using appropriate additives are theoretical approaches which could be used to enhance the stability of biological products [8,9]. Polysaccharides such as sucrose [10], trehalose [11] and sorbitol [12], and neutral proteins such as Bovine Serum Albumin (BSA) [13] are some widely-used additives in increasing the half-life of biological prod-

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ucts. If proteins, including antibodies, are not placed in the original environment, they quickly become unstable [14]. In general, instability in antibodies is a consequence of two different types of stress shocks: physical and chemical. Chemical shocks are processes or factors leading to elimination or breakage of the covalent bonds in molecular structure [15]. Deamidation is considered as the most common route for chemical degradation of proteins, peptides and antibodies [15,16]. Generally, any protein or peptide containing Asn-Xaa sequence is prone to deamidation over time [17].

In physical instability, the chemical composition of antibody remains stable, but its physical state is changed. This type of instability includes denaturation, aggregation, deposition, and absorption [8,9]. Inappropriate buffer, temperature and PH conditions are the most important factors that lead to physical instability [18]. Loss of the antibodies activity along with microbial contamination is the main side effect of keeping antibodies at room temperature [19]. Type of storage containers is another factor that has an effect on increasing or decreasing the stability of antibodies [20,21]. Keeping antibodies in solid form (Lyophilized) [22-24], storage at temperatures below 4*°C*, using cleaned glass containers [20,21], and the application of various additives [25,26] are recommended for decreasing the environmental effects on the reactivity of antibodies. To prevent the growth of microbial contaminants, antimicrobial agents such as sodium azide (NaN3) or thimerosal can be used [27].

"Lyophilization" means that the residual moisture should be less than 1 percent. Lyophilization process itself can expose proteins to a number of stressful and potentially destabilizing processes such as protein adsorption on the ice surface [28]. Increasing moisture is another interference factor, resulting in reduced stability [29]. However, overdrying protein is harmful to the stability and moderate humidity is optimal to preserve antibody structure [30]. For this purpose, during long term storage of biological compounds including antibodies, various additives are used in order to maintain the desired humidity conditions. Stabilization by means of polysaccharides can be explained using "water replacement" hypothesis [31]. Similar to water molecules, polysaccharides form hydrogen bonds with proteins and by replacement of the lost water, the original structure would be maintained and the formulation during storage is stabilized [32]. To employ antibodies for a long time structural and functional integrity should be preserved. Various additives are added to the formulation during production and storage processes in order to protect antibodies against damage. In this study, the effects of different additives on the specific reactivity of mAbs against rHBsAg were investigated. Obtained results indicated that additives such as disaccharides (sucrose, trehalose) and BSA have considerable effects on maintaining the stability of antibodies in liquid and solid phases during storage. These additives can be considered as proper components in reducing background signal noise in designing ELISA kits.

Materials and Methods

The effects of different additives on the specific reactivity of mAb (S3)

To evaluate the effects of additives on the reactivity of S3 mAb against conformational epitopes of rHBsAg, the liquid phase was selected. Environmental factors such as the material of storage container, moisture and oxygen content in the air that has considerable effect on molecular structure of antibody were considered constant in all studies.

The heat shock at 37*°C* was used for assessment of the effect of storage time on the reactivity of antibody. One week at 37*°C* is equivalent to the amount of shock that antibodies would receive in a period of one year at 4*°C* [33]. According to extensive studies on the effect of different additives on keeping the structure of proteins, 0.25 *M* sorbitol [25], 1 *M* glycerol [26], 1 *M* trehalose [26], 0.25 *M* trehalose [34], 0.04 *M* trehalose [35], 1 *M* sucrose [26,36], 0.25 *M* sucrose [10] and 0.5% BSA [13,26] were added as probable formulation stabilizers. Concentration of antibodies in all samples was considered equal to 300 *ng* per *ml*. The S3 mAbs in the presence of different additives were incubated for 12 days at 37*°C*. After the time point of 6-day and 12-day, the reactivity of antigen binding sites were evaluated. All studies were repeated three times and evaluated in two independent trials. The reactivity of mAbs was evaluated using an indirect ELISA test. The recombinant HBs antigens (serotype adw) with concentration of 0.5 *ng/ml* were coated in high protein-binding capacity polystyrene ELISA plates (Nunc). After blocking with skim milk, S3 mAbs which were affected by heat shock in the presence of different additives were used as the second layer with final concentration of 300 *ng/ml*. Then, conjugated sheep (Fab) 2 anti-mouse antibody (Sigma) was used as a detector layer. Eventually, ortho-phenylenediamine (OPD) substrate was added. After stopping the reaction using 20% sulfuric acid, absorbances were read at 492 *nm* wavelength by ELISA reader. All results were analyzed using SPSS software version 21 by Mann-Whitney nonparametric statistical test at the confidence interval of 95%.

The effects of different additives on the specific reactivity of mAbs (S7 and S11)

Among the mAbs recognizing linear epitopes, S7 and S11 antibodies were selected [37]. The obtained results from homemade ELISA kit showed that these antibodies had the most efficiency in solid phase as capture layer [6]. Therefore, the effects of additives on the reactivity of these antibodies were evaluated in solid phase. Moisture is another factor affecting the function of antibodies that are coated to solid phase [25]. In order to reduce the effect of moisture on antibodies, moisture scavenger was added to the storage container.

Similar to previously mentioned section, S7 and S11 mAbs were coated to ELISA plates at final concentration of 300 *ng* per *ml* in the presence of various additives. Then, the plates were treated by heat shock at 37*°C*. After time points of 6-day and 12-day, the stability of antibodies were evaluated using sandwich ELISA test. Briefly, the HBsAg (serotype adw) at final concentration of 64 *ng* per ml was added to ELISA plates. Then, biotin conjugated polyclonal antibodies against serotype adw at optimal dilution were added as the next layer. Strep avidin-HRP (Sigma) was applied and OPD substrate was added eventually. After stopping the reaction with sulfuric acid 20%, optical density was read at the wavelength of 492 *nm*. Similar to the previous section, Mann-Whitney test was used to analyze data.

Results

Figure 1 presents the protective effect of different additives on the specific reactivity of antigen binding site of S3 antibody after 6 days of co-incubation. This data revealed that 0.5% BSA has the most protective effect (p=0.046). The results of adding other additives were not statistically significant but 0.25 *M* sucrose, 0.04 *M* trehalose had obviously the highest protective effects after BSA. Figure 2 reveals the results of these assessments after a twelve-day-incubation. Our finding revealed that 0.5% BSA, 0.25 *M* sucrose and 1 *M* trehalose had the greatest protective effects, but the differences were not statistically significant.

Figure 3 shows the effects of different additives on the specific reactivity of mAbs (S7 and S11) against rHBsAg in the solid phase as a capture antibody after 6 days of incubation. It revealed that 1 *M* sucrose showed the most protective effect (p=0.048). The effects of 0.5% BSA, 0.25 *M* trehalose and 0.25 *M* sorbitol were considerable but they were not statistically significant.

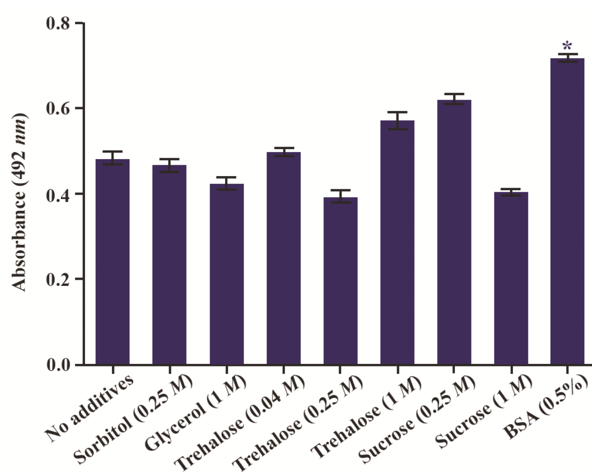

Figure1. Effects of various additives on the specific reactivity of mAb (S3) against conformational epitope after 6 days of incubation at 37*°C*. The cross reactivity between the capture layer and the detector layer as backgrounds were subtracted from all obtained ODs. The significance level is indicated with star (*) [*p<0.05].

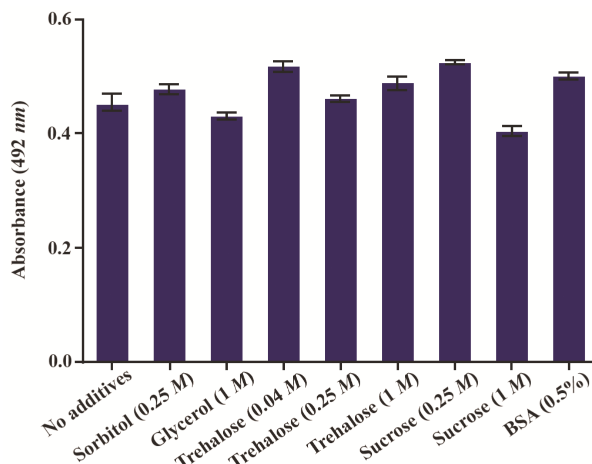

Figure 2. Different effects of various additives on the specific reactivity of mAb (S3) against conformational epitope after 12 days of incubation at 37*°C*. The cross reactivity between the capture layer and the detector layer as backgrounds were subtracted from all obtained ODs.

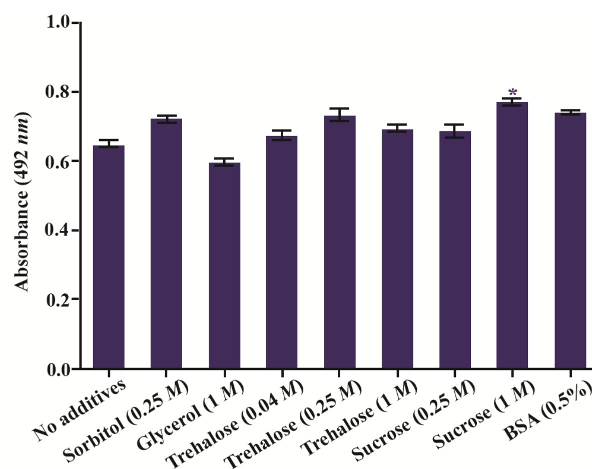

Figure 3. Different effects of various additives on the specific reactivity of mAbs (S7 and s11) against linear epitope after 6 days of incubation at 37° C. The cross reactivity between the capture layer and the detector layer as backgrounds were subtracted from all obtained ODs. The significance level is indicated with star (*) [*p<0.05].

Figure 4 demonstrates the same results after 12 days. After 12 days, all additives except glycerol showed a considerable protective effect on the reactivity of mAbs. Based on these results, 0.25 *M* sucrose and trehalose 0.04 *M* had the greatest protective effects on the efficiency of mAbs (p=0.005). The protective effects of BSA 0.5% (p=0.05), 0.25 *M* sorbitol (p=0.048), 1 *M* trehalose and 1 *M* sucrose (p=0.046) were statistically significant. 1 *M* glycerol was found to be the least efficient additive.

Discussion

Today, biological components are widely used in diagnosis and treatment and so preserving the function during storage is one of the most intrinsic aspects in biological researches [12]. Proper physical conditions and

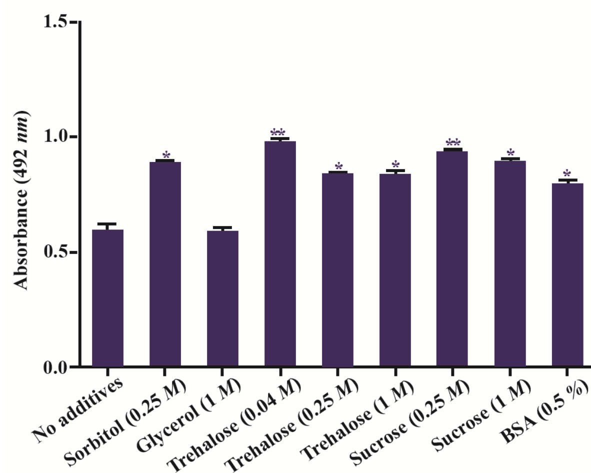

Figure 4. Different effects of various additives on the specific reactivity of mAbs (S7 and s11) against linear epitope after 12 days of incubation at 37°*C*. The cross reactivity between the capture layer and the detector layer as backgrounds were subtracted from all obtained ODs. The significance level is indicated with star (*). P-values lower than 0.05 are depicted as * and p-values lower than 0.01 are depicted as ** [*$p<0.05$, ** $p\leq 0.01$].

suitable additives are widely used in order to increase the bioactivity of antibodies [8,9]. Previous researches demonstrated that polysaccharides such as sucrose [10], trehalose [11] and sorbitol [12] and neutral proteins such as bovine serum albumin [13] increase the half-life of antibodies. Previously, different mouse mAbs against rHBsAg were produced [7] and they were used to design a sensitive and specific homemade ELISA kit [6]. Produced antibodies were divided into two categories; antibodies recognizing the conformational epitopes and antibodies recognizing the linear epitopes [7]. Although this homemade ELISA assay had acceptable sensitivity (0.5 *ng/ml*) and specificity (98%), the stability of kit was not satisfactory due to instability of produced antibodies. In order to increase the half-life of kit and decreasing the background signal noise, it was decided to study the effects of different additives on the specific reactivity of antigen binding site of mAbs.

The assessment was done at 37°*C* to decrease the time of study. Based on previous studies, the shock that antibody receives at 37°*C* for 3 days is almost equal to the same shock that it receives after 6 months of incubation at 4°*C* [33]. According to the fact that the applied shock during our assessments was heat shock, the reduction property of materials had no significant effect on maintaining the biological activity of antibodies [38].

Apart from applying biochemical additives such as polyols and polysaccharides [10] which were used in the present study, other preservative were also used [9]. Suitable additives decrease the harmful effect of physical conditions on the specific reactivity of antibodies such as denaturation, aggregation, deposition, and absorption [8,9].

In this study, polysaccharides such as sucrose and trehalose had the most efficiency in decreasing the background signal noise and improving the specific reactivity of antibodies. The effect of these components could be explained on the basis of "water replacement" hypothesis [31]. These polysaccharides form hydrogen bonds with proteins and by replacement of the lost water, the structure of antibodies would be maintained [32]. The protective effects of sucrose in maintaining the structure of L-selectin [39], recombinant hemoglobin [40] and lysozyme [36] have been reported previously. Trehalose also has positive effect on preserving the structure of lysozyme [36] and beta-lacto globulin [41]. Sucrose and trehalose had more considerable effects on maintaining reactivity of antigen binding sites of S3 mAbs in comparison to other additives. These additives increase the negative value of free energy among the original structure and denatured protein [42,43].

Although sucrose and trehalose have positive effects on the specific reactivity of S3 antibody, the efficiency of these components is minor in comparison to S7 and S11 mAbs. It could be explained that when antibodies are coated to the solid phase, a particular relative stability is gained [29,44]. For as much as in liquid phase antibody bears the highest peripheral shock, 0.5% BSA would be the most convenient additive to sustain the reactivity of S3 mAbs. Analyzing the data of S7 and S11 mAbs proved that all additives used in this study except 1 *M* glycerol have considerable effects on keeping the specific reactivity.

Better performance of additives in solid phase brings two different scenarios into mind; first, antibodies could possess a relative stability in the solid phase which might be a consequence of reducing peripheral shocks against antibodies. Secondly, additives function more efficiently in retaining the specific reactivity against antigen binding sites of linear epitopes.

In general, the major role of polysaccharides [45] and in particular disaccharides such as sucrose [10], trehalose [11,34] and sorbitol [12] is protecting antibodies against dehydration which is related to hydrogen bindings between sugar and protein molecules. This property of polysaccharides leads to an increase in the negative value of free energy between original structures and denatured proteins [42,43]. Sucrose prevents the denaturation of proteins and changes folding [10,36]. On the basis of the obtained results, it was concluded that adding sucrose and trehalose can have a major protective effect on specific reactivity of S3 mAb. Although the effect of these selected additives on S7 and S11 mAbs was comparably shown to be minor but it should be kept in mind that even this amount of preservation could be effective in decreasing the background signal noise.

Conclusion

It was concluded that sucrose, trehalose and BSA have considerable effects on the specific reactivity of anti rHBsAg mAbs during long storage. Application of

these additives at different dilutions is recommended in the formulation of MAbs production.

Acknowledgement

This study was financially supported by a grant from Tehran University of Medical Sciences, Tehran, Iran.

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