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Polysaccharides as protectants for paper-based analytical devices with antibody

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ABSTRACT

Paper with immobilized protein can provide a low-cost platform for diagnostics, but evidence is emerging that the instability of protein on paper during shipping and storage severely limits its commercial development. Of these, paper-immobilized antibody, though widely used, is in urgent need of improvement in the shelf life. Herein we provided a detailed investigation of a simple and versatile approach for achieving stable antibody on paper to improve bioassay performance. Anti-Blood Group A IgM was chosen as the model antibody and its activity was tested via agglutination reactions. Representative polysaccharides (i.e., chitosan, sodium alginate and dextran) were used as protectants and their ability to stabilize antibody under heat and desiccation stresses was investigated. It was found that the presence of dextran with high concentration markedly stabilized paper-immobilized IgM for at least 120 days. Our results indicate that dextran with good film-forming ability could be a protectant for paper-immobilized antibody.

Graphic abstract

Keywords: dextran, antibody immobilization, paper-based biosensor, polysaccharide protection

1. Introduction

Protein, as sensitive biological compounds, has an increased tendency to lose function under some external stress such as heat, radiation, desiccation and shearing [1-3]. It usually requires temperature-controlled supply chains to retain biological activity in the pharmaceutical and biotech industries, resulting in cost increase and use limitation [4,5]. Formulations for protein storage and stability have been developed; freeze-drying is the most common strategy to preserve protein in the form of dehydrated powders [6]. Such powders are typically used to extend the shelf life of products, but some types of protein still can be damaged during the process [7,8].

The development of many experimental approaches and applications in biological and chemical sciences has brought about an increasing requirement for the immobilization of protein onto solid substrates [9,10]. Protein immobilized on various supports also needs to be protected to perform the biological functions. Among them, paper has demonstrated excellent properties as a novel support, including low cost, portability and feasibility of patterning via inkjet printing [11,12]. Many studies reported the potential of protein-based paper biosensors as simple point-of-care devices, but at the same time highlighted the need for protein stabilization strategies [13,14].

As an important type of protein, antibody, also known as immunoglobulin, plays a crucial role in current bioactive paper-based diagnostics [15-17]. The storage and stabilization of paper-immobilized antibody is a basic concern to evaluate the performance of these bioassays. The work by Ramachandran et al. and the preliminary study in our group have shown that freeze-dried antibody immobilized on paper could be stored for a long period of time at room temperature without significant loss of the bioactivity [18,19]. However, the freeze-drying process is not a cost-effective way for the large-scale manufacturing of bioactive paper with immobilized antibody, which, in turn, makes itself not suitable to be a generic approach.

Different from antibody protection, enzyme, as another commonly used type of protein for bioactive paper, has been successfully protected on paper by several easy and inexpensive approaches in the past few years. Alkasir et al. observed that tyrosinase enzyme, entrapped between chitosan and sodium alginate layers deposited onto filter paper, could show 92% residual activity after 260-day storage [20]. Zhang et al. reported that glucose oxidase and lactate oxidase could retain full activity over 6 months of storage on starch-coated paper [21]. Therefore, currently antibody is in urgent need of a simple, low-cost and robust solution of its bioactivity protection. Encouraged by the above-mentioned successful application of natural polysaccharides on paper-immobilized enzyme preservation, it is of great value to exam the compatibility of this cost-effective approach with paper-immobilized antibody. Meanwhile, although the studies have presented effective enzyme protection on paper via polysaccharides, there is a lack of detailed analysis about polysaccharide use, which could give more accurate instructions to the fabrication of bioactive paper.

Of the five classes of immunoglobulins (IgA, IgD, IgE, IgG and IgM), IgG and IgM are by far the most frequently used antibodies in bioassays, and they share a similar structure and function [22]. Considering IgM is more susceptible than IgG to denaturation, Anti-Blood Group A IgM with great use was chosen as a model antibody in our study. These agglutinating antibodies have been widely used to fabricate paper-based analytical devices for point-of-care diagnostics. Rapid blood typing can be performed on paper by using agglutinating antibodies to induce red blood cell (RBC) agglutination [23,24]. Besides, plasma separation based on RBC agglutination has been proved to be useful for designing paper-based bioassays for detecting plasma components like glucose [25]. Thus the long-term stability of antibody on paper is important for improving bioassay performance. Herein, various polysaccharide strategies of immobilizing IgM on paper were investigated, including polysaccharide covering, supporting and blending. The important factors involving

polysaccharide modification and IgM immobilization were analyzed by agglutination reactions. The results provide an easy and inexpensive path to prolong the shelf-life of antibody-based paper biosensors and serve as valuable references for the implementation of paper-based biosensing systems.

2. Materials and methods

2.1 Reagents

Low-molecular-weight chitosan (Cat. No. 448869, 20-300 cP, 1% in 1% acetic acid), low-molecular-weight sodium alginate (i.e., NaAlg, Cat. No. A0682, 4-12 cP, 1% in H_2O) and low-molecular-weight dextran (Cat. No. D1662, ~ 4 cP 10% in H_2O [26]) were purchased from Sigma-Aldrich. Anti-Blood Group A IgM (abbreviated as IgM in this study) for further manufacturing use (FFMU) purchased from Commonwealth Serum Laboratory (CSL), Australia was used as the model antibody. Red blood cells (RBCs) from CSL Revercell™ (15 % A1 and 15% B RBCs) were concentrated to 45% before use. Kleenex paper towel (Kimberly-Clark, Australia), as the paper substrate, was cut into 1 cm × 1 cm pieces to immobilize antibodies. All reagents were used without further purification and all solutions were prepared using ultrapure water (18 MΩ·cm, Mill-Q Gradient System, Millipore).

2.2 Instrumentation

Dark field images of papers were obtained by an Olympus BX60F microscope. Fluorescence images of fluorescein isothiocyanate labelled bovine serum albumin (FITC-BSA) and RBCs labelled with FITC were captured with a Nikon confocal microscope. SEM images were recorded with a FEI Nova NanoSEM™ scanning electron microscope. Water Drop Penetration Time (WDPT) test was carried out by a contact angle instrument (Dataphysics OCA230, Germany) as follows: A paper square was mounted onto the sample

holder, and a 3 μL ultrapure water droplet was delivered onto the paper surface by a glass syringe. Then the penetration time of the water droplet through the paper was calculated from the video of the water drop penetration process. The test was repeated three times at room temperature. The images of agglutination assays (see below for details) were recorded by a desktop scanner and quantitative analysis was achieved using ImageJ software to invert and measure the grayscale intensity of blood spots. Error bars (standard deviation) were obtained from three repeats of the test.

2.3 Red Blood Cell Agglutination Assays

The interaction of IgM with RBCs can have two possible results: agglutination, referred as positive assay (+), and non-agglutination, referred as negative assay (-). Kleenex paper towel that has larger paper pore size than filter paper possesses an enhanced ability to elute non-agglutinated RBCs, although Kleenex paper towel and filter paper have comparable ability to fix agglutinated RBCs [27]. Thus Kleenex paper towel was chosen as the paper substrate in this study. The color intensity value could be obtained by subtracting the measured intensity of the negative from that of the positive in the grayscale mode (0–255). Successful testing should easily distinguish the positive assay from the negative one; that is, the positive assay should report a well-defined blood spot on paper while non-agglutinated RBCs from the negative assay can be easily washed out of the paper by 0.01 M pH 7.4 phosphate-buffered saline (PBS) (Fig. 1A and 1B). So a high color intensity value would be obtained. In order to perform the bioactivity evaluation of paper-immobilized IgM, both 3 μL 45% antigen-positive and antigen-negative reagent RBCs (i.e., A1 and B RBCs) were respectively introduced onto two testing paper squares immobilized with IgM, followed by 90 s incubation and then 2× 50 μL PBS washing. If the bioactivity of IgM is not retained on paper, the positive assay could not report a well-defined blood spot, but falsely show the result as a

negative one (Fig. 1C). . Therefore, we would get a low color intensity value. The value of color intensity declined with the decrease of paper-immobilized IgM activity. Obviously, agglutination assays as an indicator provide a simple and semi-quantitative method for evaluating the bioactivity of paper-immobilized IgM.

2.4 Antibody Immobilization on Papers with Different Polysaccharide Formulations

Polysaccharide covering: 5 μL of IgM was spotted on the paper squares and dried for 30 min. Then 5 μL of chitosan solution in aqueous acetic acid with 0.9% NaCl, NaAlg solution in PBS and dextran solution in PBS were respectively applied to the paper squares, followed by 30 min drying at room temperature. With this method, IgM spotted on paper surface was covered by polysaccharides (Fig. 2).

Polysaccharide supporting: 5 μL of chitosan, NaAlg and dextran solution were applied to the paper squares and allowed to dry for 30 min, respectively. Then 5 μL of IgM was spotted on each of the freshly prepared papers and dried for 30 min. With this method, IgM was spotted on a polysaccharide layer that supported the IgM and reduced the contact between IgM and paper (Fig. 2).

Polysaccharide blending: 5 μL of IgM solution containing chitosan, NaAlg or dextran were respectively applied to the paper squares, which were then dried for 30 min. With this method, IgM was incorporated into the polysaccharide matrix (Fig. 2). The activity of immobilized IgM was evaluated by the agglutination reactions as mentioned above.

2.5 Thermal and Storage Stability Studies of Different Papers with Immobilized Antibody

During heating studies, the freshly-prepared paper samples were aged in an oven at 60 °C for various incubation periods. For long-term stability studies, all paper samples were prepared as previously described, placed in pill containers under ambient conditions, and

stored at room temperature in the dark. Residual bioactivity of paper-immobilized IgM was evaluated using agglutination tests.

3. Results and discussion

3.1 Concentration-dependent Antibody Bioactivity

The knowledge of paper-immobilized antibody stability can strongly help to reduce the cost while sustaining the effectiveness of paper bioassays. So we first investigated the impact of antibody concentration on the stability of paper-immobilized IgM when stored at room temperature in the dark. The serial dilution data show that IgM, although being diluted, retained its activity at day 0 (Fig. 3). However, the loss of IgM activity was observed at all storage concentrations within 10 days. The original IgM (protein concentration ranging from several mg/mL to tens of mg/mL) had the relatively low rate and extent of deactivation, while activity loss was comparatively obvious for diluted IgM. As shown in the inset image in Fig. 3, there is no significant color difference between positive and negative results for paper samples with 50-fold and 100-fold diluted IgM, causing low intensity values. The results were consistent with previous reports that many proteins could display greater stability at higher concentration [28]. The obvious deactivation of IgM only after being stored for 10 d for the whole dilution-series suggests proper storage demands antibody stability on paper. However, Fig. S1 shows that the dilution-series of IgM via dextran blending strategy retained its activity within 10 days. Thus it is of great value to perform a detailed analysis of polysaccharide protection for paper-immobilized IgM. Considering the commonly used concentration range of paper-immobilized protein (tens of to hundreds of μg/mL) and agglutination assay responses to the dilution-series of IgM, a 1:10 dilution of IgM was used throughout this study, unless specially noted.

3.2 Polysaccharide Modification and Antibody Immobilization

Inspired by successful biomolecule immobilization via layer-by-layer assembly technique for many applications [29,30], herein we proposed three simple types of antibody immobilization strategies: covering, supporting and blending, which were classified based on the role played by polysaccharides during the process of antibody immobilization. Representative polysaccharides, i.e., cationic chitosan, anionic NaAlg and neutral dextran, were chosen for antibody immobilization. On one hand, cellulosic fibers have a porous structure (1-30 nm micropores) [31]. Due to the nanoporosity of cellulosic fibers, polysaccharides with molecular weights of several thousands could penetrate into the fiber wall, which may preclude the effective antibody-polysaccharide interactions. On the other hand, polysaccharides with high molecular weight cannot be applied onto paper easily and cannot provide effective protection for proteins via intermolecular interactions due to steric hindrance [32], so polysaccharides with relatively low molecular weights (up to tens of thousands) were utilized. Moreover, an increase of polysaccharide concentration would obviously increase the viscosity of the polysaccharide solution, resulting in the difficulty of sample loading onto paper. In practice, chitosan and NaAlg were used with the concentration of no more than 2% w/v while concentrated dextran solution (up to 40% w/v) was employed. We believe that the differences in the compositions and structures of polysaccharides determine the different operating ranges of concentration. Besides hydroxyl groups, amino groups in chitosan and carboxyl groups of NaAlg can also form hydrogen bonds with hydroxyl groups of polysaccharide chains respectively, which could cause the relatively high viscosity of polysaccharide solutions.

Dark field microscopy and SEM were used to characterize the nature of polysaccharide coatings on paper. Many polysaccharides were reported previously to have the good film-forming ability because of their intra and intermolecular hydrogen bonds [33]. High-concentration dextran (i.e., 40%), as shown in Fig. 4, could supply enough polysaccharide to

fill the pores and voids between the fibers, thereby developing a thin film on the paper surface. By contrast, chitosan and NaAlg at 2% concentration only interpenetrated the fiber network, generated evenly distributed coatings on surface of paper fibers and did not obviously change the surface porosity of paper. Then the morphology and distribution of immobilized antibody via different strategies was examined by using 1 mg/mL FITC-BSA as a model protein. Fig. 4 and Fig. S2 show that FITC-BSA could be relatively well distributed on the paper. Compared with the inset images, it was found that proteins with different immobilization strategies could penetrate almost throughout the paper network and were relatively homogeneous in the direction of lateral solution movement.

3.3 Antibody Performance on Different Papers

An ideal immobilization strategy should not do damage to the functionality of biomolecules, and therefore the effect of employing various polysaccharide formulations for IgM immobilization on paper was initially evaluated by performing RBC agglutination assays. All three chitosan-based immobilization methods, as shown in Fig. 5, were proved to fail to perform effective agglutination assays. It was therefore not suitable to evaluate the protective power of chitosan to antibody molecules via agglutination assays. Such behaviour is related to electrostatic interactions between the positively-charged protonated amine on chitosan chains and negatively-charged RBC membrane. When treated with chitosan, RBCs lost their typical biconcave morphology and coalesced into a clot that could not be easily washed off paper sheet by PBS buffer (Fig. S3). Thus no significant difference was observed between positive and negative testing, which resulted in the low intensity value.

Unlike chitosan, different immobilization approaches using NaAlg or dextran display different results. Among the three strategies, the most effective RBC agglutination was observed via polysaccharide-blending immobilization strategy (Fig. 5), and the color intensity

was found in the increasing order of covering < supporting < blending strategies. Despite a lack of electrostatic attraction like chitosan, NaAlg and dextran can still act as a physical shield to restrict RBC access to the active sites of IgM when choosing the covering method, which in turn indicates the limitation of polysaccharide-covering strategy as a generic approach for immobilizing biomolecules on paper because of potential interference with bioassays. However, the other two strategies enabled IgM to react with RBCs more efficiently than the control (paper-immobilized IgM without polysaccharides), as polysaccharides with certain viscosity could reduce the flow rate and facilitate the interaction between the immobilized IgM and RBCs. The latter strategy (blending) was better than the former (supporting) since blending could promise a sufficient contact between IgM and RBCs.

3.4 Paper-immobilized Antibody Storage

To evaluate the application potential of polysaccharide as paper-immobilized antibody stabilizer, we compared the stability of IgM under different conditions. As mentioned above, IgM that was deposited on paper without any polysaccharide protection denatured rapidly and lost its function (Fig. S4). Fig. 6 and Fig. S5 show that IgM deposited on paper by the supporting and blending strategy in the presence of NaAlg or dextran has the improvement in shelf life. For polysaccharide solutions at the same concentration, the signals from the supporting strategy decreased more rapidly than those from the blending strategy during 2-month storage. Irreversibility of polysaccharide adsorption to cellulose has been reported because of the similarity in their chemical structures [34]. Unlike the homogeneous blending, we believe that the supporting strategy makes polysaccharide unable to dissolve sufficiently to engage in the interactions with IgM effectively when adding IgM on polysaccharide-coated paper, which is not good for the protection of IgM on paper. The blending method, on the

other hand, delivered a relatively satisfying signal within 2 months. Even with the low concentration, a detectable signal was still observed at day 60, indicating that some of antibody was still active (Fig. 6). These results confirmed that polysaccharide could extend the shelf life of antibody deposited on paper.

Stability improvements via blending strategy became much stronger in the comparison of supporting and blending in the two polysaccharide systems; stabilization of antibodies blended with polysaccharide was proved to be dependent on polysaccharide concentration: color intensity increased with increasing concentration for NaAlg and dextran (Fig. 6). During dehydration and storage, IgM blended with polysaccharides could bring about the matrixes with high viscosity and low molecular mobility. The entrapment of IgM in dried polysaccharides with an amorphous state prevents the unfolding of antibody molecules. As can be seen in Fig. 6A, NaAlg, although only small amounts were used, still could provide a viscous environment for IgM preservation. In terms of dextran, higher concentration resulting in higher viscosity and more IgM-dextran interactions prevented IgM denaturation. Notably, when the dextran dosage in the blend was increased to 40%, about 85% initial potency of IgM was retained after 2 months of storage at room temperature (Fig. 6B) and paper-immobilized IgM shows ~ 80% residual activity after 120-day storage (Fig. S6). These data indicate that the presence of dextran film is important for the improvement in stabilization of paper-immobilized IgM. As mentioned above, when 40% dextran solution resolidified into films upon drying, protein molecules were well distributed throughout the dextran film matrix. In this case, the entrapment of IgM into a biocompatible dextran film with good dispersion performance could effectively prevent IgM from going through conformational changes during storage.

Besides paper bioactivity, paper wettability, which determines the penetration of liquid samples or reagents into the paper sheet, is another important property that affects the performance of paper-based bioassay. We believe that a moderate level of wettability is favorable for bioactive paper, which may enhance the interaction between liquid samples or reagents and paper-immobilized biomolecules. In this study, antigens on the surfaces of RBCs and IgM in the polysaccharide layer require time to interact. This interaction is dependent upon the dissolution of polysaccharide matrices and mixing of RBCs with released IgM. A moderate wettability of bioactive paper could allow time for those events to occur. In contrast to this, very low paper wettability is not conducive to antigen-antibody interaction, since low wettability suggests that slow dissolution of polysaccharide might be encountered, which could cause incomplete or even no assay development because of insufficient paper wetting. As can be seen in table S1, an increased storage time can reduce the wettability of papers with immobilized IgM, which can be attributed to the exposure of the hydrophobic regions of the antibody, promoting intermolecular interactions leading to aggregation. Since protein denaturation could cause an increase in the surface hydrophobicity, we therefore expected a significant improvement of paper wettability via 40% dextran blending that could offer efficient antibody protection. The data of the water drop penetration time supports our reasoning, and the paper with 40% dextran blending strategy has a moderate level of wettability after storage that could be useful for preforming paper-based bioassays. The high stability of IgM in the dextran film provided effective support for the retention of hydrophilicity of paper with immobilized IgM.

3.5 Thermal Stability of Antibody Immobilized on Paper

Obviously, the limited refrigeration resources, particularly in developing countries, also promote demand for good thermostabilization of paper-based biosensors; that is, paper-

immobilized biomolecules should be relatively stable under continuous hot weather exposure. Hence the heat stability of paper-immobilized IgM was evaluated after exposure to 60 °C for various heating times. As shown in Fig. 7A, IgM in aqueous solution with and without polysaccharides lost its activity by heating at 60 °C for 1 h. Because the degradative processes of protein could be facilitated in the presence of water [35], different concentrations of NaAlg or dextran cannot offer enough protection for IgM molecules in aqueous solutions against heat denaturation.

Interestingly, paper-immobilized IgM displays different levels of stability when tested under acute heat stress conditions. Even in the absence of polysaccharides, only a moderate loss (~ 40%) of IgM activity was observed after an eight-hour heat treatment (Fig. 7B). Considering the structural relevance of IgM and IgG, the results are in reasonable agreement with those obtained by Wang et al., where they reported paper-immobilized IgG were relatively stable at 80 °C and lower [16]. We think that the immobilization of antibody onto paper can reduce thermal movement, which in turn prevents antibody denaturization through aggregation. IgM on paper protected by polysaccharides shows similar profile of activity loss to IgM on paper without protection, but having a reduced magnitude. Polysaccharides therefore provide protection against thermal inactivation for paper-immobilized IgM. Both NaAlg and dextran can suppress the heat-induced inactivation of paper-immobilized IgM in a concentration-dependent manner. The highest recovery of IgM activity was observed in the presence of the highest concentration of NaAlg (2%) and dextran (40%); approx. 20% and 15% activity loss after 8-hour heating, respectively. Such protection could be related to the inhibition of structural alterations in antibody. Polysaccharides like NaAlg and dextran, having a relatively high glass transition temperature in the dry state, can inhibit molecular mobility and consequently enhance IgM thermostability on paper (Fig. S7). Pronounced stabilization of IgM in dextran film at the elevated temperature suggests this method could

also provide sufficient thermal stability to paper-based biosensors. The concentration of dextran has less effect on thermal stability than on storage stability. We believe that it may be associated with the different rates of dehydration under different conditions. Dextran film provides an inexpensive and effective protection environment without the use of vacuum and desiccant. Moreover, unlike chitosan and NaAlg, natural polymeric carbohydrates like dextran, having hydroxyl groups as the primary functional groups, is very hydrophilic and non-charged, which could be benign to typical bioassays [36,37].

4. Conclusions

In this work, the application area of polysaccharide protection was extended and dextran was applied as an effective formulation component for enhancing stability of antibody attached to paper. Appropriate coating system (blending) could increase the shelf life of IgM compared with IgM deposited onto unmodified paper. The stabilization data presented here indicate that the formation of hydrophilic films in paper plays a vital role in protecting paper-immobilized antibody. Dextran reduced protein unfolding and aggregation at elevated temperatures, and also provided structural stability to IgM during storage. Since most studies emphasize the invention of paper-based bioassays, we believe that our results of this study will be of use for improving process, distribution and use of paper bioassays without the costly cold chain.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found in the online version. Figures S1−S7 for additional fluorescence images and antibody activity as a function of storage time. Table S1 for the data of water drop penetration time.

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Figure Captions

Fig. 1. Schematic presentation of antibody activity test via agglutination assays on paper.

Fig. 2. Schematic presentation of antibody immobilization on paper via different polysaccharide strategies.

Fig. 3. Influence of concentration on the activity of paper-immobilized IgM when stored for 0 (■) and 10 days (□). The inset image shows the color difference between paper samples incubated with A1 RBCs (+) and B RBCs (-) respectively, after 10 d storage.

Fig. 4. Imaging and characterization of paper samples. Upper row: dark filed images of different papers. Middle row: SEM images of different papers. Lower row: confocal images of papers immobilized with FITC-BSA via the blending strategy. Insets: images of the edges of the solution flow. Paper without polysaccharide modification was chosen as the control.

Fig. 5. Agglutination assays on different papers: paper-immobilized IgM with % w/v concentrations of polysaccharides (chitosan from 0.5 to 2, NaAlg from 0.5 to 2, dextran from 10 to 40); paper-immobilized IgM without polysaccharides as a control. Relative intensity was expressed as a percentage compared with the color intensity value of the control.

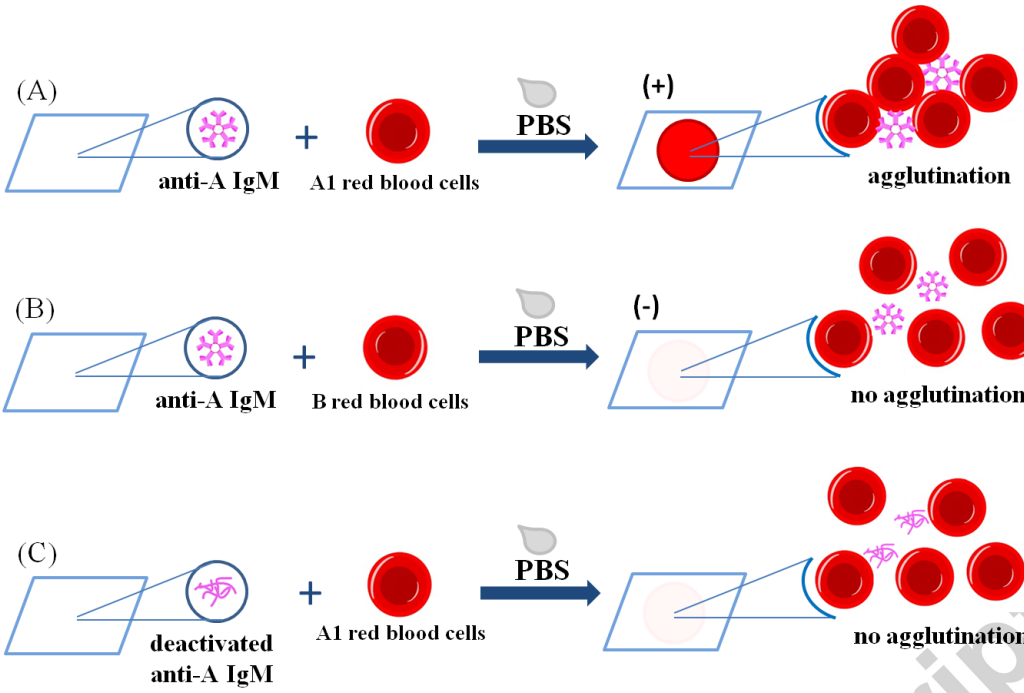
Fig. 6. IgM storage stability on different papers. (A) Paper-immobilized IgM via NaAlg blending. (B) Paper-immobilized IgM via dextran blending. Relative activity was expressed as the percentage of IgM activity that remained after storage in comparison to the activity at day 0 for each group.

Fig. 7. IgM thermal stability at 60 °C. (A) Paper-based agglutination assay of IgM solution after 1 h heating: IgM solution with or without polysaccharides (control) was heated and then

dried on paper followed by the assay. (B) Paper-immobilized IgM thermal stability as a function of time. Paper-immobilized IgM without polysaccharides as a control. Relative activity was expressed as the percentage of IgM activity that remained after incubation in comparison to the activity at hour 0 for each group.

Highlights

- Dextran as a simple and efficient protectant for bioactive paper with antibody
- High-concentration dextran markedly stabilized IgM immobilized on paper for 4 months
- Polysaccharide-based film encapsulation hold the key to antibody protection on paper
- Polysaccharide as protectants for antibody supplements bioactive paper with protein



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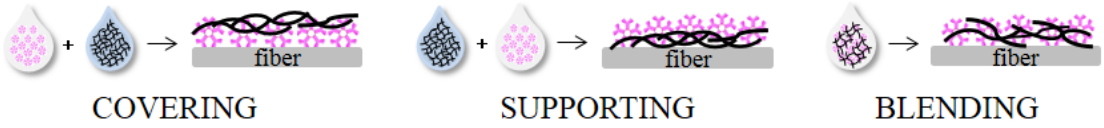

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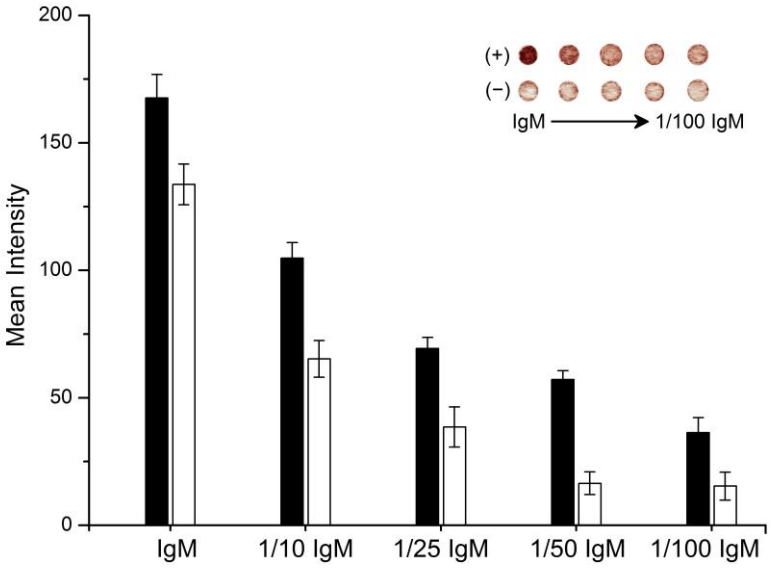

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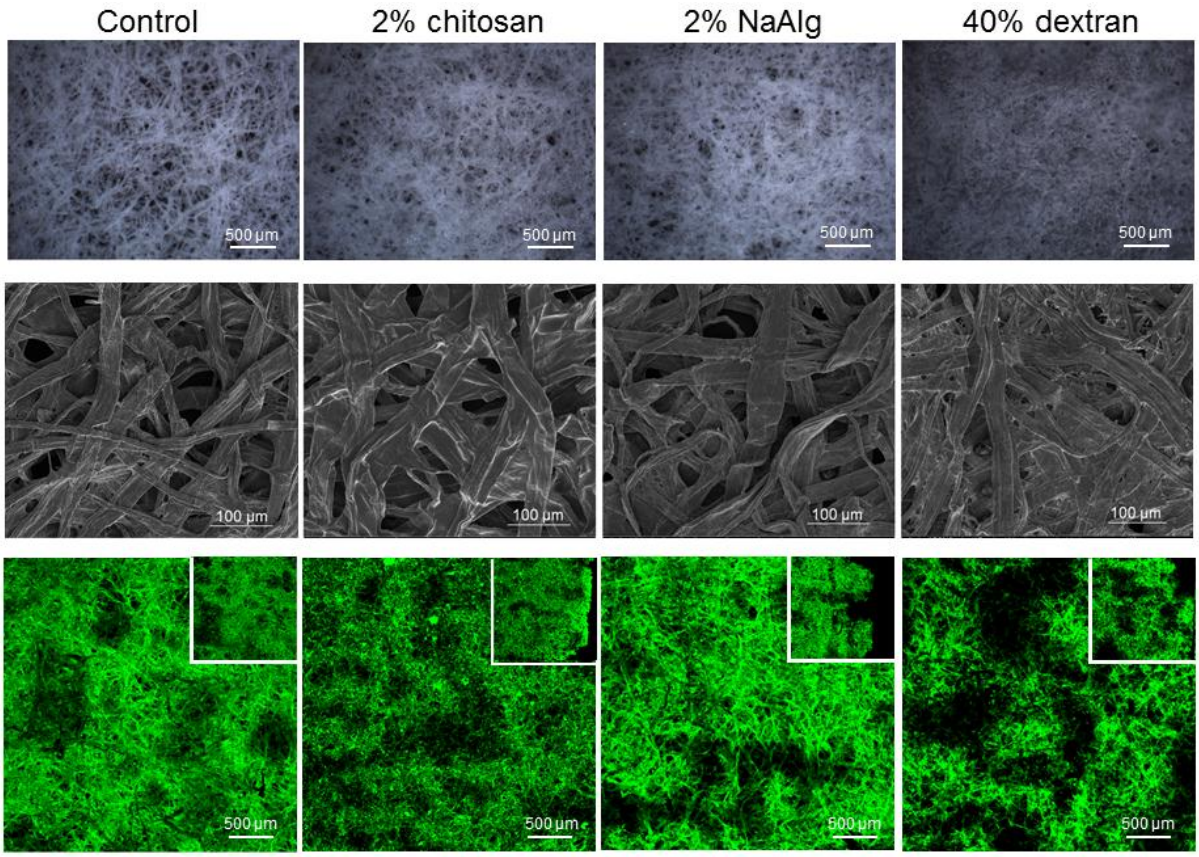

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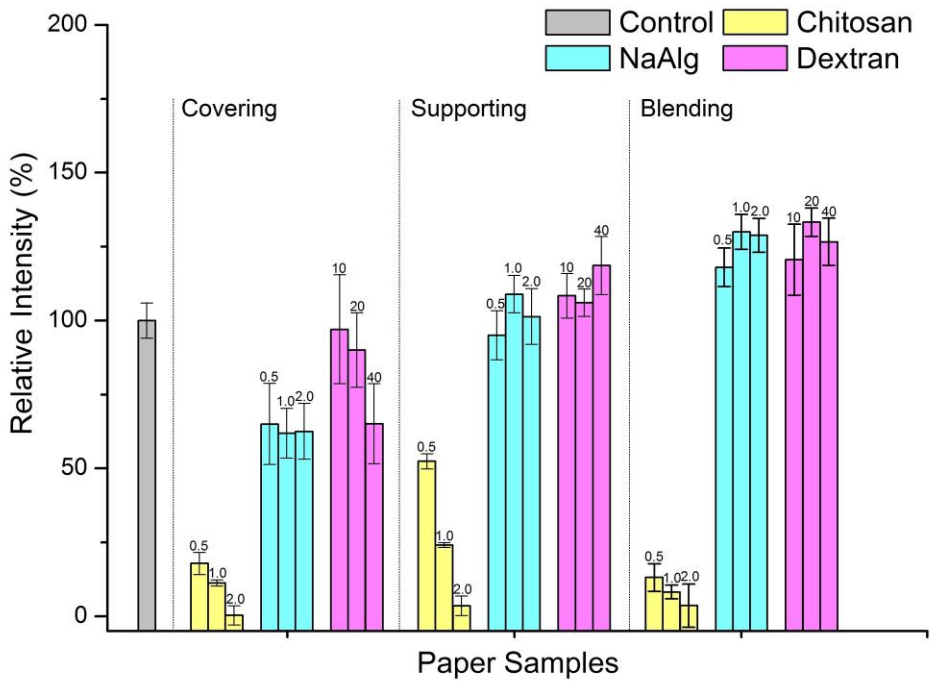

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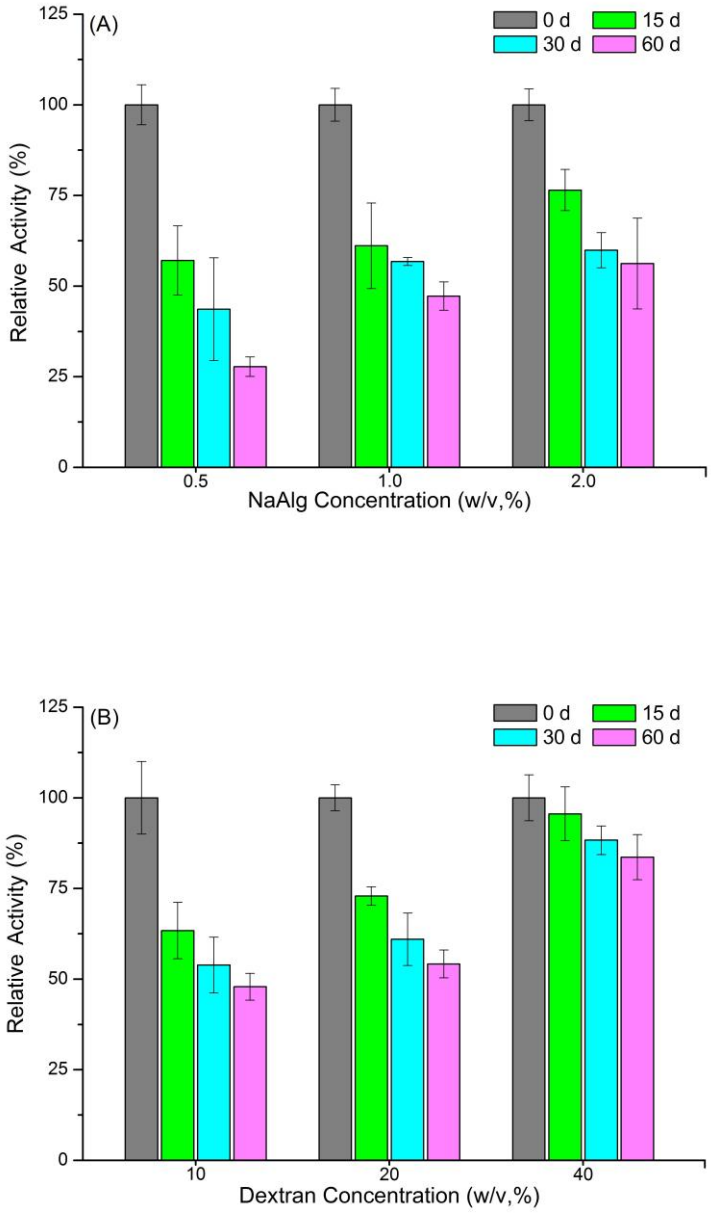

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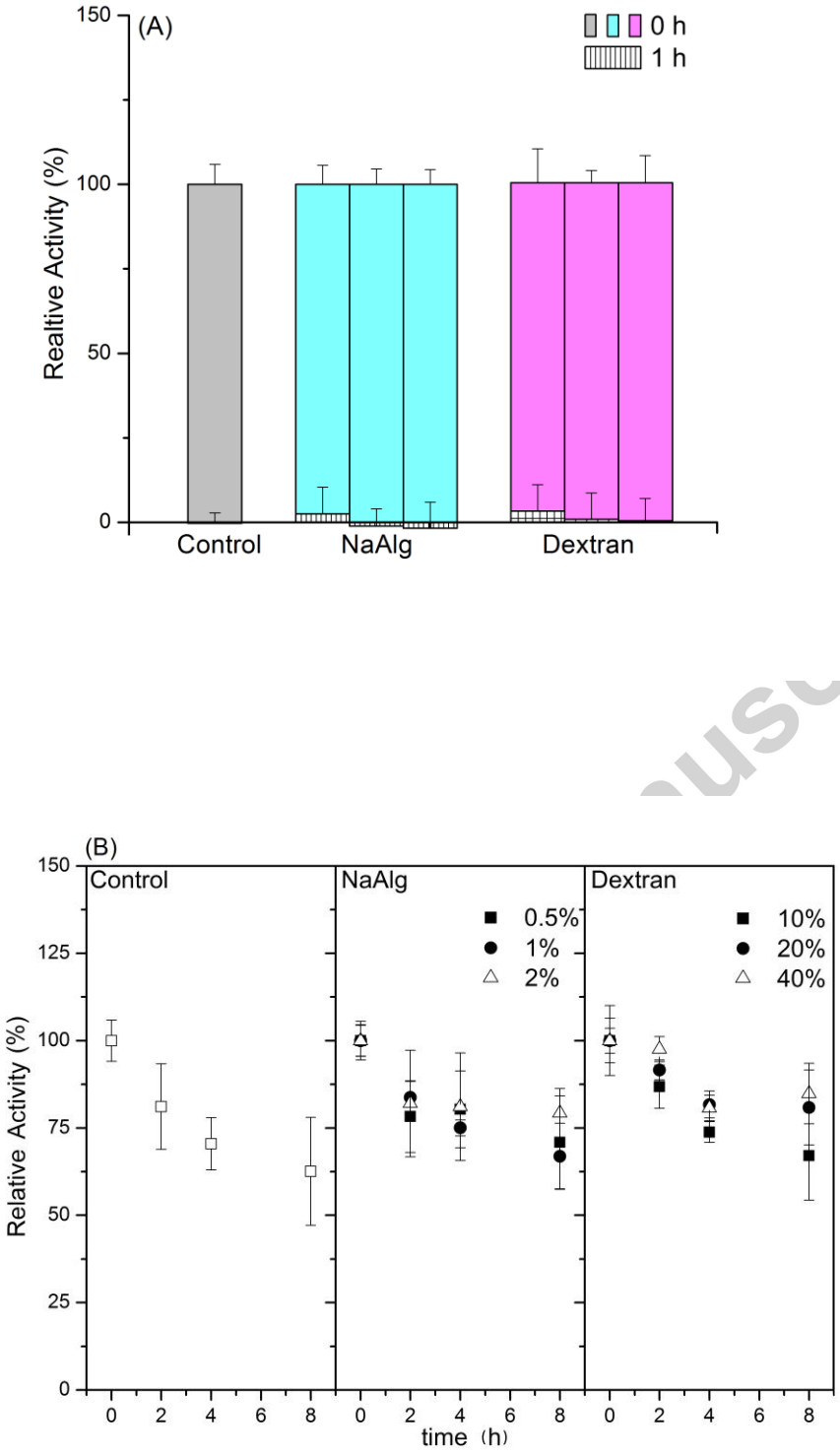

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Graphic abstract