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Anal. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.analchem.7b02447 • Publication Date (Web): 20 Jul 2017

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Rapid, Sensitive and Quantitative Detection of Anti-PEG Antibodies by mPEG-coated SPR Sensors

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Abstract

Pre-existing and induced anti-poly(ethylene glycol) (PEG) antibodies (abs) have been shown to be related with limitation of therapeutic efficacy and reduction in tolerance of several therapeutic agents. However, the current methods to detect anti-PEG abs are tedious and usually lack of quantification. A facile, rapid, sensitive and reliable technique to detect anti-PEG abs is highly desired in both research and clinic settings. In this work, we have presented a surface plasmon resonance (SPR) biosensor technique for the detection of anti-PEG abs and compared three PEG surface chemistries. Methoxy-PEG (mPEG) 5k was found to have the best performance. The detection of anti-PEG abs directly from diluted blood serum was achieved within 40 min. Detection sensitivity is as good as or better than enzyme-linked immunosorbent assay (ELISA). Furthermore, different antibody isotypes can be quantitatively differentiated by adopting secondary antibodies. A pilot study has been performed to analyze clinical blood samples using this technology, demonstrating its potential as a convenient and powerful method to pre-screen and monitor anti-PEG abs in the patients before or after they receive treatment with PEG-containing drugs.

Poly(ethylene glycol) (PEG) is the most widely used synthetic polymer in pharmaceutical industry to conjugate biological therapeutics or as formulation excipients.¹⁻³ The attachment of PEG to biomolecules such as recombinant proteins and peptides (or PEGylation) extends their circulation time, reduces immunogenicity and antigenicity and improves therapeutic efficacy.⁴ Currently, twelve therapeutic PEGylated drugs have been approved by the US Food and Drug Administration (FDA), such as PEG-asparaginase for leukemia and lymphoma and PEG-uricase for chronic gout, and more are in the pipeline.⁵

Nevertheless, in contrast to the generally accepted assumption that PEG is non-immunogenic and non-antigenic, an emerging number of studies demonstrated that the immune system can elicit antibody formation specifically against PEG (anti-PEG abs) both in animals and human.⁵⁻⁸ The generation of anti-PEG abs can accelerate the clearance of PEGylated therapeutics, thus reducing therapeutic efficacy. For instance, recent clinical investigations of PEG-uricase in refractory chronic gout patients unequivocally showed that anti-PEG abs correlated with a reduction in drug effectiveness.^{9,10} Moreover, the existence of anti-PEG abs threatened patient safety with anaphylaxis and infusion reactions.^{9,11,12} Re-exposure to PEG containing drugs may greatly increase the chance for adverse effects due to B cell memory of anti-PEG abs.¹³ To make things worse, recent findings have indicated the widespread occurrence of anti-PEG abs in general population due to increased daily exposure to PEG containing products.^{14,15} If high titer anti-PEG abs are present in blood, even people without known allergies may have severe hypersensitive reactions when receiving PEG containing therapeutics for the first time.^{11,12} Thus, screening and monitoring the anti-PEG abs in blood before and during the treatment with PEG containing drugs are of particular importance to provide safety and maintain therapeutic efficacy. A facile, rapid, sensitive and reliable technique to detect the anti-PEG abs is highly desired to fulfill the demand.

A variety of methods have been reported to detect anti-PEG abs such as serology¹⁶, flow cytometry¹⁶, Western blotting^{17,18} and enzyme-linked immunosorbent assays (ELISA)^{19,20}. However, these traditional methods usually involve time-consuming and complicated procedures, which hinder clinical applications. In addition, it is difficult to quantify the antibody concentrations using these methods, and the detection results are highly dependent on operation protocols, quality of reagents and even the personal habits of the operator. For these reasons, the detection results from different reports using these

methods are often not comparable.

To overcome these shortcomings, we proposed a novel method with high sensitivity, simple operation process, rapid detection time and high throughput using a surface plasmon resonance (SPR) sensor. SPR is a powerful technique to monitor label-free biomolecular interactions and biomolecule/surface interactions.^{21,22} Previously, we modified SPR sensor chips with poly[poly(ethylene glycol) methyl ether methacrylate] (PEGMA) polymer and demonstrated its capacity to detect anti-PEG abs qualitatively.²³ Herein, we compared three different surface coating chemistries and achieved sensitive quantitative detection with methoxy-PEG. The PEG modification simultaneously serves as dual roles: first, it resists non-specific protein adsorption from diluted blood serum and diminishes the background noise; second, it acts as an anchored antigen to catch any anti-PEG abs present in the blood sample to generate strong signals in SPR measurements. With this facile setup, quantitative anti-PEG abs detection could be achieved within 40 mins with a detection limit as low as 10 ng/mL. In addition, antibody isotypes can also be distinguished and quantified using secondary antibodies.

Results and Discussion

To effectively detect anti-PEG abs from blood samples, which constitute hundreds of different proteins together, two parameters are crucial in the design of the sensor chips. One is to strongly resist non-specific protein adsorption to ensure a low-noise background while another is to have strong binding affinity to the analyte to produce a high signal. As shown in Fig. 1, three different chemistries were selected to modify chip surfaces with PEG-derived materials and compared for their performance. Oligo(ethylene glycol) (EG4) self-assembled monolayer (SAM) is known to resist non-specific protein adsorption²⁴ and used in this work. Anti-PEG abs were reported to bind to as few as three oxyethylene groups¹⁹. Two other methoxy-terminated PEG analogs, mPEG and PEGMA were chosen instead of hydroxyl terminated counterparts due to their stronger binding affinity to anti-PEG abs.¹⁹ EG4 and mPEG were modified onto gold chip surfaces by immersing into corresponding thiol solutions, while PEGMA brushes were synthesized by surface-initiated atom transfer radical polymerization (ATRP). The coating thickness was measured by ellipsometry that the PEGMA layer was 16.5 ± 2.1 nm, mPEG layer was 4.2 ± 0.6 nm, and the EG4 SAM was 1.5 ± 0.2 nm.

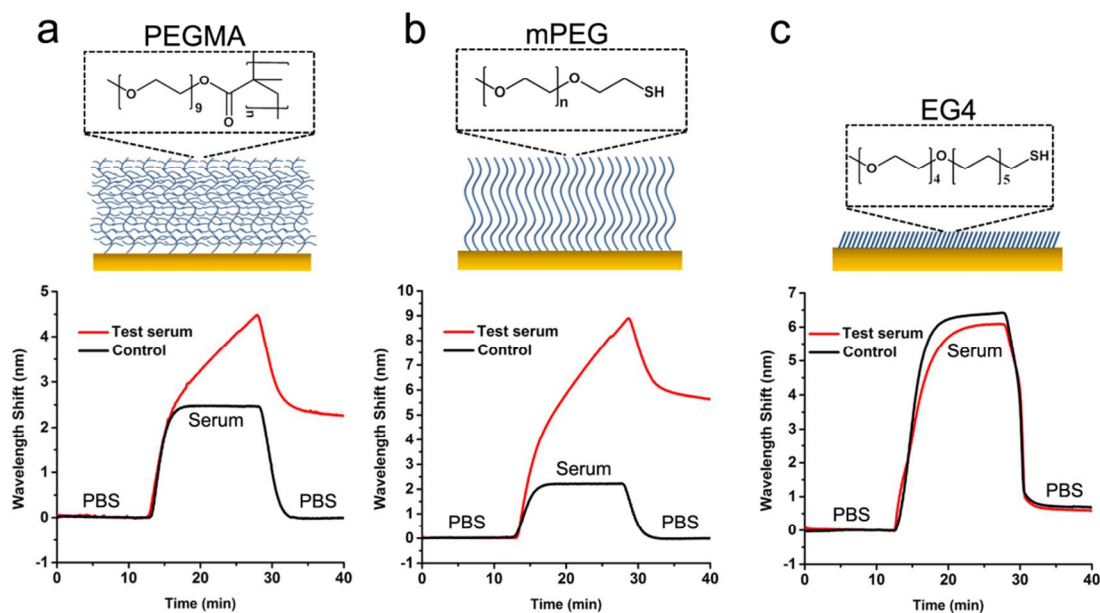


Figure 1. The gold chip surfaces were modified by PEGMA polymer brush (a), mPEG (b) or EG4 SAM (c), and tested by both negative control (black) and anti-PEG (red) serum samples.

The resistance to surface nonspecific protein adsorption from blood proteins was first examined by flowing diluted control serum. The test experiment was done by flowing 5% serum dilution for 15 min, followed by PBS washing for another 10 min. As shown in Fig. 1d-f, a large wavelength shift signal was recorded when serum samples flowed past the chip due to the solution refractive index change. After washed by PBS buffer, the remaining signal represented surface molecular adsorption. Although EG4 SAM is known to reduce nonspecific protein adsorption from single protein solutions, it still showed 0.7 nm wavelength shift biofouling signal when challenged by 5% serum dilution, which corresponded to $\sim 12 \text{ ng/cm}^2$.²¹ In contrast, no fouling signal was detected for both mPEG and PEGMA grafted surfaces. The blood sample collected from rats that received three repeated intravenous (IV) injections of uricase-PEG conjugates were used as the test sample to examine the three sensor chips. When flowing 20 times diluted test serum, mPEG modified chip showed the strongest sensing signal (5.6 nm wavelength shift), indicating its highest binding affinity against anti-PEG abs. The PEGMA grafted sensor chip exhibited moderate response (2.3 nm wavelength shift), while the signal from EG4 chip was not distinguishable from biofouling background. As expected, anti-PEG abs

have stronger binding affinity toward linear PEGs compared with branched PEGMA polymers,²⁵ as they were elicited using linear PEG conjugated proteins. mPEG was chosen as the coating material for the following detection studies due to its highest sensitivity in a low-noise background.

Selecting mPEG as the surface coating layer, we next examined the sensor response to different anti-PEG ab concentrations. As shown in Fig. 2a-b, monoclonal rat anti-PEG IgM ab was used to test the SPR sensor at a series of concentrations. As expected, the sensor signals increased with the test antibody concentrations. The detection limit was determined to be 50 ng/mL and the chip surface adsorption reached saturation when antibody solution was higher than 20 μ g/mL. As seen in the sensorgram, the background signal generated by the difference in media refractive index between PBS and sample disappeared at $\sim$ 30 min. To avoid the influence from media refractive index, we took the wavelength shift value at 35 min to represent the sensor signal for each antibody concentration. The sensor signals showed ideal linear relationship with antibody concentrations in the range of 50 ng/mL to 10 μ g/mL. Similar results were also observed for mouse anti-PEG IgG abs, with the detection limit of 100 ng/mL (Fig. 2c-d).

One intrinsic limitation for currently used antibody detection methods is their non-linearity between the signal responses and analyte concentrations. For example, as the most state-of-the-art immunoassay to quantify anti-PEG abs, ELISA uses enzyme mediated biochemical reactions to generate signals. A four parameter logistic regression is needed to correlate ELISA signals with the anti-PEG abs concentrations, as shown in Fig. S1. With the non-linear relationship, it is always difficult to directly compare two optical density (OD) results without a standard curve. As a result, the antibody titer is defined as a measurement to facilitate the semi-quantitative comparisons. To determine the titer for a blood sample, a series of sample dilutions need to be tested in parallel which significantly increases the experimental workload. In contrast, the linear relationship demonstrated by SPR analysis provides great convenience when comparing different experiments in the absence of a standard curve. As shown in Fig. 2e-f, we mixed the rat anti-PEG serum with different amount of control serum to simulate a series of different samples. The wavelength signals from serum samples also demonstrated a well fitted linear relationship, showing that the anti-PEG levels from different samples can be directly compared by SPR measurements.

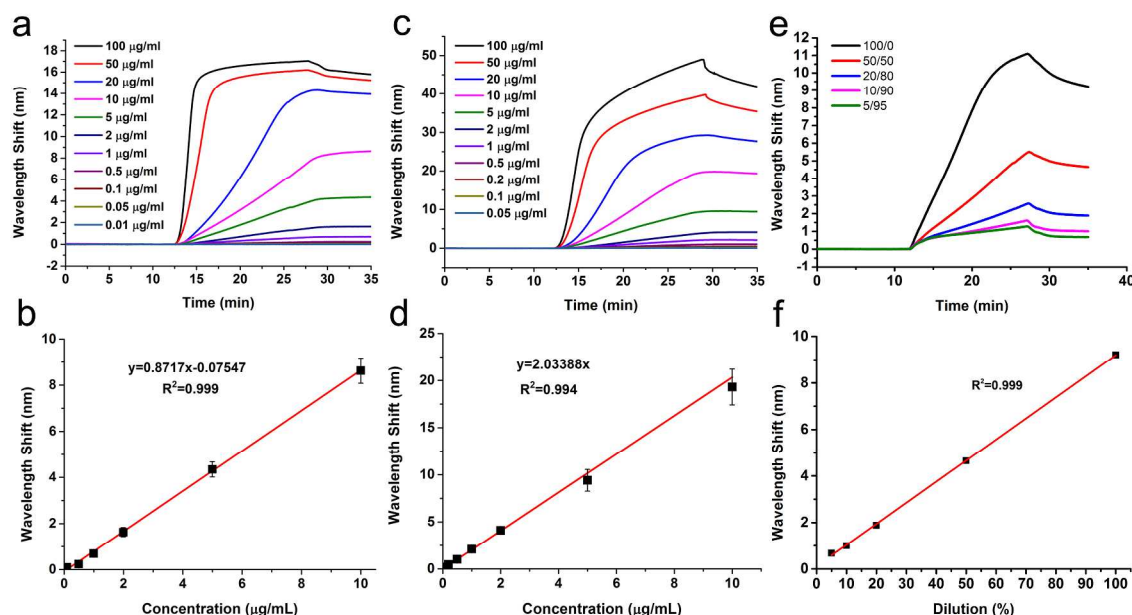


Figure 2. SPR sensorgrams of rat monoclonal anti-PEG IgM (a,b) and mouse monoclonal anti-PEG IgG (c, d) detection at different analyte concentrations. The wavelength shift values were correlated with antibody concentrations. A linear dynamic range was found as 0 to 10 µg/mL for both antibodies. (e, f) SPR sensor responses to anti-PEG abs in serum samples. An anti-PEG serum was mixed with control serum at the ratio of 100/0, 50/50, 20/80, 10/90 and 5/95 and examined by the sensor. The wavelength shift values showed excellent linear relationship with the amounts of anti-PEG abs in blood samples.

Antibodies belong to a family of immunoglobulin that consists of different varieties known as isotypes. In placental mammals there are five antibody isotypes known as IgA, IgD, IgE, IgG, and IgM, among which IgG and IgM are the most abundant antibodies present in blood circulation. The difference in their heavy chains results in altered complex structures with different molecular weights. For example, IgG appears as a monomer while IgM exists as a much larger pentamer. To accurately monitor the development of immune responses, the detection should be able to differentiate each antibody isotype quantitatively. To identify the isotypes, we adopted secondary antibodies in our experimental settings. As shown in Figure 3a, taken the detection of anti-PEG IgM as an example, the properly diluted sample was firstly flowed for 15 min,

followed by PBS washing, then the goat anti-rat IgM antibody solution (secondary antibody), and finally PBS washing. When anti-rat IgM abs bound to surface adsorbed rat anti-PEG IgM, an increase of wavelength shift showed on the sensorgram. We correlated the wavelength shifts induced by secondary antibody adsorption with a series of analyte samples, and the signal is in linear relationship with the anti-PEG ab concentrations for both IgM and IgG (Figure 3b, c). Such results demonstrated that blood anti-PEG IgM abs can be quantitatively determined in a wide range of 10 ng/mL to 10 μ g/mL and anti-PEG IgG concentrations can be measured in the range of 50 ng/mL to 5 μ g/mL. It should be noted that the application of secondary abs amplified the signals, thus increased the signal/noise ratio and extended the detection limits of anti-PEG IgM to 10 ng/mL and IgG to 50 ng/mL. By contrast, direct ELISA tests of the same sets of standard samples showed the detection limits of 100 ng/mL for anti-PEG IgM and 1 μ g/mL for anti-PEG IgG abs (Figure S1).

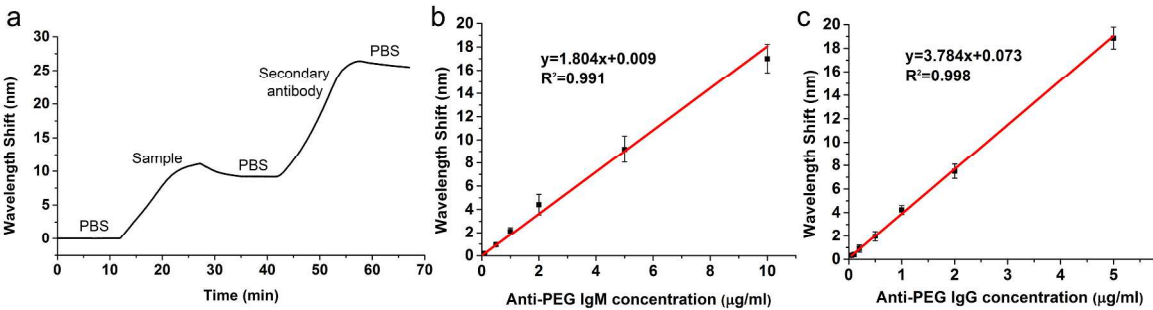


Figure 3. (a) A typical SPR sensorgram of anti-PEG antibody isotype detection using secondary antibodies. (b, c) Monoclonal anti-PEG abs were spiked into control serum at different concentrations to prepare standard samples. The wavelength shift signals from secondary antibodies were correlated with concentrations of standard samples to generate standard calibration curves for rat anti-PEG IgM (b) and mouse anti-PEG IgG (c).

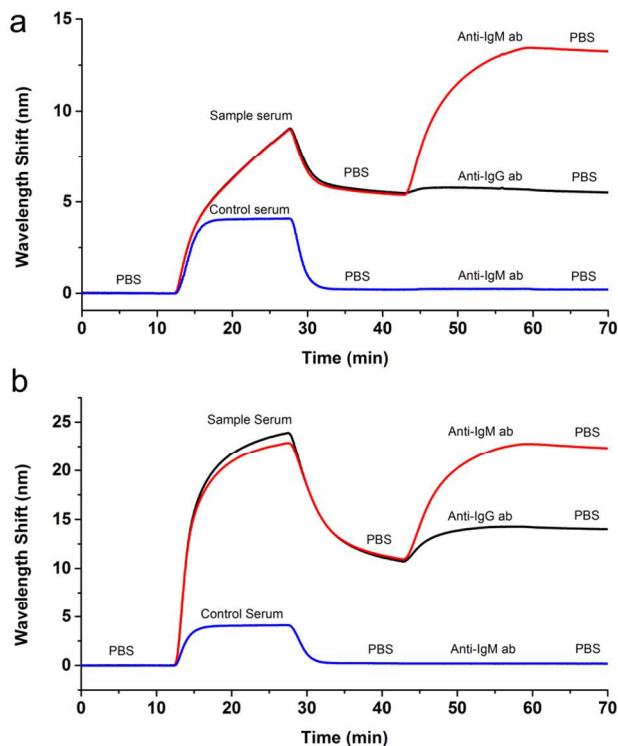


Figure 4. SPR sensorgrams of complete anti-PEG ab detections from rat blood samples. (a) Anti-PEG serum generated by IV immunization and (b) anti-PEG serum generated by SC immunization with Freund's adjuvant.

With the established detection protocol and standard curves generated using monoclonal anti-PEG antibodies, the concentration of each anti-PEG ab isotype can be quantitatively detected. To demonstrate its application to real circumstances, we generated and examined two different anti-PEG antisera from rats to simulate clinical blood samples. One serum was obtained by immunizing a rat with three weekly intravenous (IV) injections of uricase-PEG conjugates, while the other was made by immunizing a rat using uricase-PEG conjugates mixed with Freund's adjuvants subcutaneously (SC). Freund's adjuvant is known to be very effective in stimulating cell-mediated immunity and leads to the production of certain immunoglobulins and effector T cells. As shown in Figure 4, both serum samples generated strong signals, indicating the abundance of anti-PEG abs in these sera. The wavelength shift when flowed SC sample was much larger than that of IV sample, demonstrating the fact that SC immunization with Freund's adjuvant is much more efficient in generating antibodies. Using the wavelength shift

values from secondary anti-IgM abs, we quantitatively determined that the anti-PEG IgM concentrations were 4.3 $\mu\text{g/mL}$ for IV sample and 6.3 $\mu\text{g/mL}$ for SC sample, respectively. Due to lack of availability of the monoclonal rat anti-PEG IgG antibody standard, we were not able to quantify the absolute concentrations of anti-PEG IgG in these serum samples. However, from the wavelength shift values (0.1 nm for IV and 3.3 nm for SC), we can draw the conclusion that SC sample contained 33-fold higher anti-PEG IgG than IV sample, due to the stronger responses and faster anti-PEG isotype switching stimulated by Freund's adjuvant.

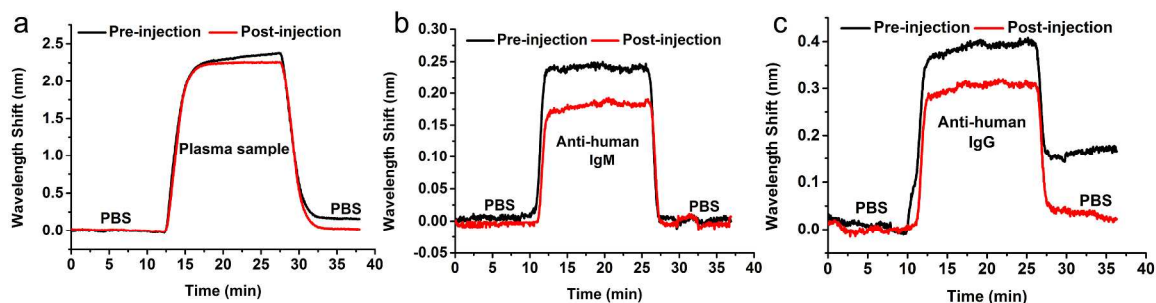


Figure 5. Plasma samples from one leukemia patient who received PEG-asparaginase therapy were analyzed by a SPR sensor. (a) sensorgram when flowing plasma samples; (b) sensorgram when flowing anti-human IgM secondary ab; and (c) anti-human IgG secondary ab.

The goal of this study is to provide a convenient high-throughput technology for clinical anti-PEG ab detections. To explore its translational feasibility, we did a pilot study that involves clinical plasma samples from a leukemia patient who received PEG-asparaginase therapy. The study was approved by the Institutional Review Board of the Seattle Children's Hospital. It should be noted that there is no difference in applying serum or plasma as the testing liquid. The patient had no known PEGylated therapeutic exposure history. However, adverse reactions were observed during the fourth administration of PEG-asparaginase. We analyzed two plasma samples from this patient: one was taken before PEG-asparaginase administration and the other was taken ten days post the fourth drug administration. As shown in Figure 5a, a clear anti-PEG ab response was observed for the pre-injection sample, demonstrating the existence of pre-existing anti-PEG abs. In contrast, the sensorgram showed negative response for the post injection

sample, which is possibly because these antibodies bound to the injected PEG-asparaginase and formed immune complexes. The isotype differentiation study demonstrated the pre-existing anti-PEG abs were mainly IgGs. These samples were validated by ELISA tests, and an anti-PEG IgG titer of 1:400 was found for the pre-injection sample. With these analysis data, we could surmise that the pre-existing anti-PEG IgG might be related with the adverse reactions¹⁶, although a definitive conclusion could only be made based on a larger sample size.

Conclusions

In conclusion, we demonstrated a simple, rapid, sensitive and reliable technique to directly detect anti-PEG abs in blood samples using SPR sensors. Three PEG derived materials have been tested as the chip coating, and mPEG demonstrated the best analyte signal without fouling background under detection conditions. Compared with traditional methods, the linear response of SPR sensor to anti-PEG ab concentrations greatly facilitates the comparison among different blood samples. By adopting the secondary antibodies in the detection settings, we were able to differentiate and quantitatively determine the concentration of different anti-PEG antibody isotypes in blood samples. This detection method can be readily developed into a high throughput automatic setting, which holds great potential in both academic and clinical applications.

Supporting Information

Experimental Section

ELISA calibration curves of standard monoclonal anti-PEG abs

Acknowledgments

This work was supported by the Defense Threat Reduction Agency (HDTRA1-13-1-0044), National Institutes of Health (1R21EB020781-01), and National Science Foundation (CBET 1264470). Clinical blood samples were kindly provided by Seattle Children's Hospital.

References

- (1) Mitragotri, S.; Burke, P. A.; Langer, R. *Nat. Rev. Drug Discov.* **2014**, *13*, 655-672.
- (2) Harris, J. M.; Chess, R. B. *Nat. Rev. Drug Discov.* **2003**, *2*, 214-221.

- (3) Kolate, A.; Baradia, D.; Patil, S.; Vhora, I.; Kore, G.; Misra, A. *J. Control. Release* **2014**, *192*, 67-81.
- (4) Alconcel, S. N. S.; Baas, A. S.; Maynard, H. D. *Polym. Chem.* **2011**, *2*, 1442.
- (5) Garay, R. P.; El-Gewely, R.; Armstrong, J. K.; Garratty, G.; Richette, P. *Expert Opin. Drug Deliv.* **2012**, *9*, 1319-1323.
- (6) Zhang, P.; Sun, F.; Liu, S.; Jiang, S. *J. Control. Release* **2016**, *244*, 184-193.
- (7) Yang, Q.; Lai, S. K. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* **2015**, *7*, 655-677.
- (8) Sherman, M. R.; Williams, L. D.; Sobczyk, M. A.; Michaels, S. J.; Saifer, M. G. *Bioconjugate chem.* **2012**, *23*, 485-499.
- (9) Hershfield, M. S.; Ganson, N. J.; Kelly, S. J.; Scarlett, E. L.; Jaggars, D. A.; Sundy, J. *S. Arthritis Res. Ther.* **2014**, *16*, R63.
- (10) Lipsky, P. E.; Calabrese, L. H.; Kavanaugh, A.; Sundy, J. S.; Wright, D.; Wolfson, M.; Becker, M. A. *Arthritis Res. Ther.* **2014**, *16*, R60.
- (11) Ganson, N. J.; Povsic, T. J.; Sullenger, B. A.; Alexander, J. H.; Zelenkofske, S. L.; Sailstad, J. M.; Rusconi, C. P.; Hershfield, M. S. *J. Allergy Clin. Immunol.* **2016**, *137*, 1610-1613.
- (12) Povsic, T. J.; Lawrence, M. G.; Lincoff, A. M.; Mehran, R.; Rusconi, C. P.; Zelenkofske, S. L.; Huang, Z.; Sailstad, J.; Armstrong, P. W.; Steg, P. G.; Bode, C.; Becker, R. C.; Alexander, J. H.; Adkinson, N. F.; Levinson, A. I.; Investigators, R.-P. *J. Allergy Clin. Immunol.* **2016**, *138*, 1712-1715.
- (13) Longo, N.; Harding, C. O.; Burton, B. K.; Grange, D. K.; Vockley, J.; Wasserstein, M.; Rice, G. M.; Dorenbaum, A.; Neuenburg, J. K.; Musson, D. G.; Gu, Z.; Sile, S. *Lancet* **2014**, *384*, 37-44.
- (14) Yang, Q.; Jacobs, T. M.; McCallen, J. D.; Moore, D. T.; Huckaby, J. T.; Edelstein, J. N.; Lai, S. K. *Anal. Chem.* **2016**, *88*, 11804-11812.
- (15) Chen, B. M.; Su, Y. C.; Chang, C. J.; Burnouf, P. A.; Chuang, K. H.; Chen, C. H.; Cheng, T. L.; Chen, Y. T.; Wu, J. Y.; Roffler, S. R. *Anal. Chem.* **2016**, *88*, 10661-10666.
- (16) Armstrong, J. K.; Hempel, G.; Koling, S.; Chan, L. S.; Fisher, T.; Meiselman, H. J.; Garratty, G. *Cancer* **2007**, *110*, 103-111.
- (17) Ishida, T.; Ichihara, M.; Wang, X.; Yamamoto, K.; Kimura, J.; Majima, E.; Kiwada, H. *J. Control. Release* **2006**, *112*, 15-25.
- (18) Wang, X.; Ishida, T.; Kiwada, H. *J. Control. Release* **2007**, *119*, 236-244.
- (19) Saifer, M. G.; Williams, L. D.; Sobczyk, M. A.; Michaels, S. J.; Sherman, M. R. *Mol. Immunol.* **2014**, *57*, 236-246.
- (20) Liu, Y.; Reidler, H.; Pan, J.; Milunic, D.; Qin, D.; Chen, D.; Vallejo, Y. R.; Yin, R. *J. Pharmacol. Toxicol. Methods* **2011**, *64*, 238-245.
- (21) Ladd, J.; Zhang, Z.; Chen, S.; Hower, J. C.; Jiang, S. *Biomacromolecules* **2008**, *9*, 1357-1361.
- (22) Vaisocherova, H.; Faca, V. M.; Taylor, A. D.; Hanash, S.; Jiang, S. *Biosens. Bioelectron.* **2009**, *24*, 2143-2148.

- (23) Zhang, P.; Sun, F.; Tsao, C.; Liu, S.; Jain, P.; Sinclair, A.; Hung, H.-C.; Bai, T.; Wu, K.; Jiang, S. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, 12046-12051.
- (24) Li, L.; Chen, S.; Zheng, J.; Ratner, B. D.; Jiang, S. *J. Phys. Chem. B* **2005**, *109*, 2934-2941.
- (25) Qi, Y.; Simakova, A.; Ganson, N. J.; Li, X.; Luginbuhl, K. M.; Ozer, I.; Liu, W.; Hershfield, M. S.; Matyjaszewski, K.; Chilkoti, A. *Nat. Biomed. Eng.* **2016**, *1*, 0002.

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