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Development of a portable device for the detection of anti-PEG antibodies in human plasma

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ABSTRACT

The conjugation of biological drugs with polyethylenglycole (PEGylation) is widely used to reduce their immunogenicity, thereby enhancing the safety and efficacy of the drugs. For instance, some extended half-life drugs for hemophilia A patients are PEGylated FVIII products. Unfortunately, it was observed that PEG itself, whether derived from PEGylated drugs or present in everyday products (such as cosmetics or medical devices) may induce the production of anti-PEG antibodies, resulting in hypersensitivity reactions and/or loss of efficacy of PEGylated drugs. There is therefore a demand for cost-effective and rapid point-of-care (POC) measurements. To this end, a POC device based on the principle of surface plasmon resonance (SPR) is here presented. The sensor chip is functionalized directly with PEG chains via thiol chemistry and inserted into a holder manufactured in 3D printing technology. The functionalization process has been optimized to recognize anti-PEG antibodies (both IgG and IgM isotypes) and characterized using X-ray Photoelectron Spectroscopy (XPS) and contact angle (CA) measurements. Dose-response curves were obtained using commercially available antibodies, both in buffer solution and in diluted human serum, achieving a limit of detection (LoD) in the nanomolar range. The reusability of the optical chip was also evaluated and, as proof of concept, plasma from control subjects and hemophilia A patients treated with PEGylated FVIII were measured, comparing the results with standard techniques such as a commercial SPR instrument and an enzyme-linked immunosorbent assay (ELISA). The results were obtained within 15 minutes from 10-fold diluted human plasma, paving the way for an easy-to-use, small-sized, portable, and low-cost POC device capable of detecting anti-PEG antibodies.

Introduction

Poly(ethylene glycol) (PEG), is a hydrophilic molecule based on repetitive units of ethylene oxide with a molecular weight range from 200 to 50000 of Da, which can be synthesized in a linear structure or branched or stars and coupled to different molecules mainly through lysine residues and the N-terminal amino group [1].

Due to the low immunogenicity of PEG [2], many substances for biomedical applications, e.g., biological drugs and vaccines, utilize PEG for shielding them from recognition by the immune system, improving the agent's pharmacokinetics and pharmacodynamics, and for making therapeutic agents more soluble.

Despite the clear advantages associated with PEGylation of drugs and nanocarriers, cases of PEG-related immunogenicity have been described [3], affecting the efficacy and safety of PEGylated molecules - by accelerating drug clearance or by inducing hypersensitivity reactions [4–7].

Several diseases base their therapeutic treatment on PEGylated compounds, one of them is hemophilia. Hemophilia A is a genetic disorder caused by missing or defective factor VIII (FVIII), a clotting protein. The extended half-life treatment is based on FVIII replacement with recombinant FVIII often conjugated with PEG chains to increase its circulating half-life [8–11] but evidence of the inefficacy of PEGylated FVIII products has been reported both as consequences of vaccination [12,13] and preexistent anti-PEG antibodies [14]. Gaballa and co-workers recently reviewed the prevalence of anti-PEG antibodies in different subjects [15], highlighting that they were found also in subjects never exposed to PEGylated drugs, and possibly related to the use of everyday products, such as cosmetics and food preservatives that can contain PEG or PEG-derivatives.

The general concentration range of anti-PEG antibodies is between 0.5 □g/mL to 120 □g/mL (3.33 – 800 nM) for IgG and lower for IgM [16] and several techniques have been used to detect the presence of anti-PEG antibodies [17]. Among them, the most commonly used technique is the enzyme-linked immunosorbent assay (ELISA) [3–6,9,12,13,16,18,19] followed by flow-cytometry [18,20]. Although ELISA is a well-established laboratory procedure, it is time-consuming, requires specialized personnel, it is highly dependent on operation protocols, quality of reagents, and even the personal habits of the operator. These aspects are common to standard laboratory techniques and represent a limitation in the point-of-care analysis. Another method has been proposed based on Surface Plasmon Resonance (SPR) [21–23]: in the work of Zhang and coworkers [24], the PEG-based molecules are immobilized directly on the sensor surface acting as an anchored antigen to catch anti-PEG antibodies present in the blood samples and also serving as "non fouling" layer, to avoid nonspecific protein adsorption from diluted blood serum, reducing in this way the background noise. Even if, SPR sensing platform offers significant advantages over the ELISA assay, such as the ability to perform label-free, real-time detection tests by applying the sample (appropriately diluted) directly to the sensing surface without the need for further processing, the data analysis associated with this technique require specialized personnel limiting its employment in general at a laboratory scale.

All the above methods do not allow to obtain a rapid response at the point-of-care, which is needed in all the situations in which the evaluation of the presence of anti-PEG antibodies can guide rational clinical decisions just before or just after treatment with PEGylated drugs. In particular,

persons with severe hemophilia have a high risk of bleeding if not regularly treated [25]. Indeed, untreated severe patients experience spontaneous bleeding events in the joints or muscles needing a long extra-schedule treatment with substantial direct and indirect costs in terms of drug and lost working days [26,27]. Furthermore, as PEGylation is widely employed in the development of other therapeutic agents across a range of medical conditions, the proposed system may have broader applicability beyond haemophilia for the monitoring of PEG-conjugated drugs in other disease contexts.

In this work, we present a portable, 3D-printed SPR system based on plastic optical fiber (POF) that can be used to detect anti-PEG antibodies in 15 minutes directly at the patient site without the need of complex instrumentation. First the functionalization has been set up on flat gold surfaces, immobilizing a thiolated PEG chain directly on the gold layer. The PEG chain length has been optimized, characterizing by X-ray Photoelectron Spectroscopy (XPS) and Contact Angle (CA) measurements the functionalized surface with a short or a long incubation time. The dose-response curves have been derived, using commercially available IgG and IgM anti-PEG antibodies, both in buffer solution and diluted human serum. In addition, an efficient regeneration procedure was developed to reuse the same surface for multiple measurements. Finally, human plasma samples from healthy subjects and hemophilia A patients, including patients exposed or not to PEGylated FVIII drugs, have been performed comparing the results with standard methods by using both commercial SPR instruments and ELISA assays.

The SPR sensor proposed in this work uses POF as a waveguide, which offers a number of advantages such as cost-effectiveness, ease of handling, and mechanical strength, making it ideal for designing miniaturized sensing chips that are easy to integrate into simple, compact, and portable setups suitable for hospital wards or decentralized clinics.

Results and discussion

Optimization of the mPEG length in the antibody recognition

Figure 1 shows the miniSPR device used for the experiments.

Figure 1: Optical point of care device used for measurements.

At first, mPEG of different lengths, ranging 400-10000 Da, were immobilized for 90 min on the optical chips. In fact, PEG molecular weight and also its shape (linear, branched and co-polymer) [24] can influence the antibody's recognition [16,21,28].

Then, these surfaces were exposed for 10 min to different concentrations of anti-PEG IgG, diluted in TBST buffer (75mM Tris-HCl, 450mM NaCl, 5mM EDTA, 0.1% w/v BSA and 0.05% v/v Tween 80 at pH 7). Figure 2 reports the results obtained highlighting the different SPR signal, and thus the different capture of anti-PEG IgG, as a function of the PEG lengths. Short or long PEGs (400 and 10000 Da) captured less antibody, while PEGs in a range between 1000 and 5000 Da resulted more useful to detect anti-PEG IgG. Previous studies showed that the PEG lengths >700 Da are required for interacting with the antibodies [28], whereas the low interaction at high molecular weight could be related, in our case, to the limited tail of the evanescent field that resulted in a signal loss for interaction sites too far from the gold surface.

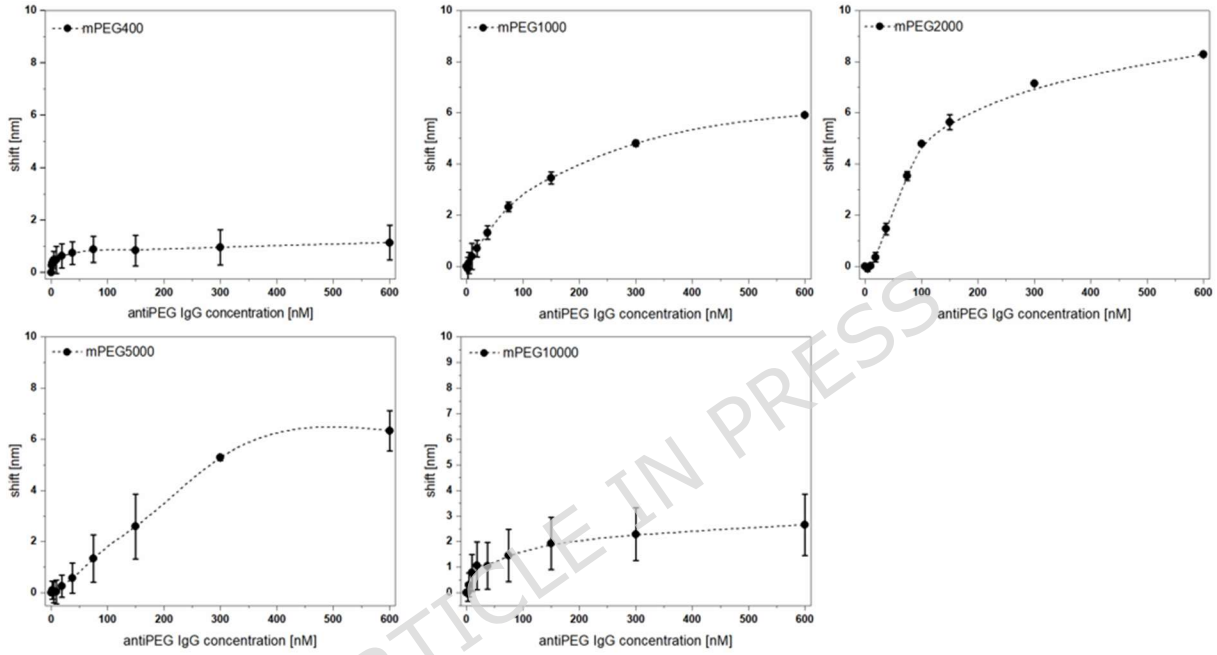


Figure 2: Dose-response curves of anti-PEG IgG on chips prepared with PEGs of different lengths. The PEG self-assembled monolayer (SAM) is incubated for 90 minutes. Data are reported as the mean value of three different chips, and error bars represent the standard deviation.

The curves shown in Figure 2 were also analyzed by applying Eqs. 2 and 3 (reported in Materials and Methods paragraph), in order to obtain the sensitivity at low concentrations and the LoD. The results of this analysis (Table S1) confirmed the better performance of the mPEG2000, which was therefore selected for the following analysis.

We then found that an overnight incubation of the PEG-SAM, instead of the 90 min incubation, allows to significantly reduce the non-specific binding (adsorption) of control IgGs on the chip surface (Figure S1).

A detailed physicochemical analysis of these two conditions is reported in the next paragraph.

Functionalized surface characterization

The flat gold surface functionalized or not with PEG2000 was characterized by standard surface chemical techniques, i.e., contact angle (CA) and XPS analysis, to verify the chemical changes occurring as a consequence of the functionalization. Table 1 summarizes all results obtained by the different techniques.

The chemical composition of the gold surface after argon plasma cleaning was in agreement with the literature and previous studies [29–31]. The SAM formation is indicated by a high carbon increase associated with the decrease of the gold signal (see Table 1). The detailed chemical analysis of the core lines of the elements is reported in Figure 3, while a fitting of the main core lines is reported in Table S2.

Figure 3 clearly shows the appearance of the main peak in the carbon core line due to the PEG molecule. The energy calibration is performed using the C 1s signal and setting at 286.45 eV the C2 component relative to the PEG molecule for all samples. The carbon content has been fitted with three or five components depending on the sample. The carbon on the cleaned gold surface ("Au") was mainly due to hydrocarburic contaminations and bonding with contaminant (visible in the survey spectrum like N, F and S). For PEG-SAMs, the highest contribution is instead related to PEG chains (C-O, the C2 component in the fitting, see Table S2) with binding energy of 286.45 eV [30,32,33]. The oxygen peak is fitted with one or two components: the first one at about 532.7 eV attributable to O-C bond in the PEG chains and the other one at 530.6 eV attributable to O=C bond.

By using the attenuation in the gold signal, it was furthermore possible to determine the SAM thickness for the two different incubation time [30], whose values are reported in Table 1, in agreement with the literature [30,34]. The CA measurements confirmed the gold substrate modification, showing an increment in the angle value from <5° (cleaned gold) up to about 35° for SAMs prepared (last column of the Table 1), in good agreement with other studies [29,30].

samples	O (%)	C (%)	Au (%)	Thickness [nm]	Contact angle [°]
Au	28.7	16.7	54.6	-	< 5
Au+ mPEG2000 90'	30.2	48.8	21	1.66	34.6 ± 6.3
Au+ mPEG2000 on	30.3	49.1	20.6	1.74	34.6 ± 2.8

Table 1. Chemical characterization determined by XPS analysis and water contact angles on cleaned gold (Au) or on mPEG2000 SAM on gold after a short incubation (90 min) or a longer one (overnight). XPS standard error does not exceed the 1–2% of the reported value.

A) B)

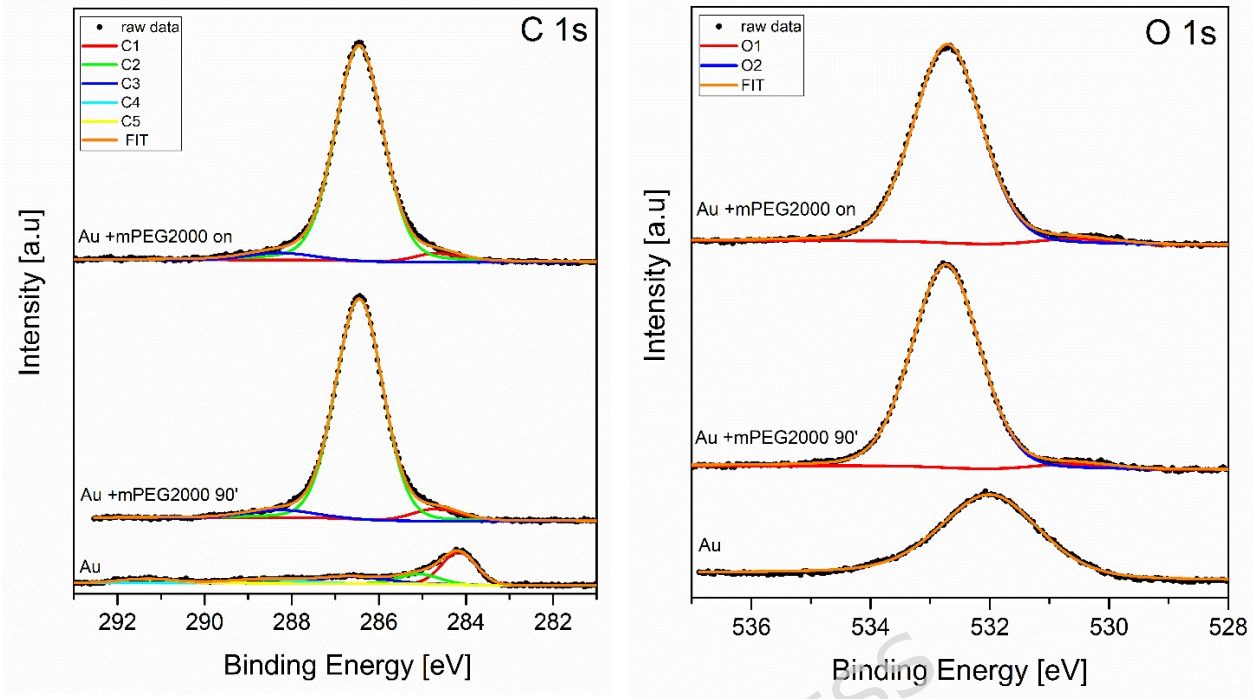


Figure 3: Detailed core lines C 1s (A) and O 1s (B) for the different surfaces. "Au": bare gold surface after plasma cleaning. Different mPEG2000 SAMs prepared in 90 min or overnight (on).

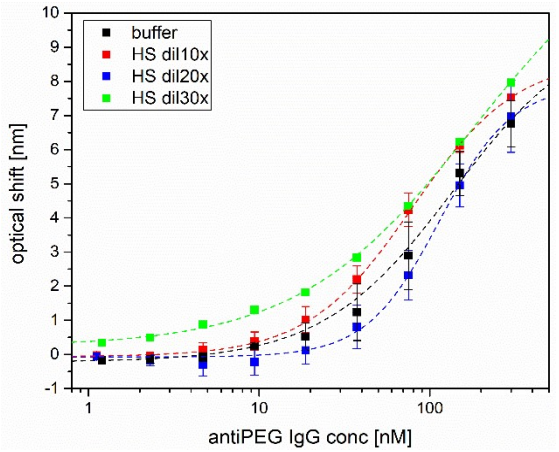
Despite no significant chemical difference was observed between the two conditions (90 min or overnight incubation), we found a significant difference in the non-fouling effect (Figure S1). This could be due to a change in the PEG brushes orientation occurring during the overnight incubation.

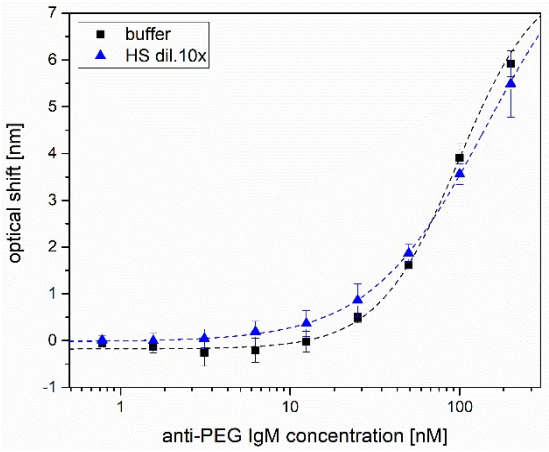
Measurements in buffer and human serum

Initial studies were carried out by spiking TBST buffer or diluted human serum with different concentrations of commercial anti-PEG IgG or IgM, ranging 1÷300 nM, and incubating these solutions on the PEG2000 surfaces. Experiments performed on a standard SPR instrument showed no difference in the performance between serum and plasma samples (Figure S3). Figure 4 shows that a concentration-dependent SPR signal was observed in all cases: the behavior between buffer and 10x or 20x diluted serum samples is quite similar considering the standard deviations while 30x diluted serum samples show a significant different behavior maybe attributable to an interference with serum component not observed at higher concentrations. However, the analytical parameters obtained from the different curves by the estimated LoD and sensitivity values (Figure 4C) show that no significant differences were observed between buffer and diluted serum, indicating that the recognition of anti-PEG antibodies is not influenced by the biological matrix.

Lower LoDs have been reported by Zhang and coworkers, who reached a LoD of 0.08 nM and 0.7 nM for IgM and IgG respectively [24] but using a commercial SPR instrument.

A B





C

	fluid	LoD [nM]	S (Sensitivity at low conc.) [nm/nM]
Anti-HUMAN IgG	Buffer	1.49	0.08
	HS dil. 30x	2.02	0.07
	HS dil. 20x	2.03	0.07
	HS dil. 10x	0.89	0.11
Anti-HUMAN IgM	Buffer	0.77	0.08
	HS dil. 10x	4.61	0.07

Figure 4: Dose-response curve of anti-PEG IgG (A) or anti-PEG IgM (B) on chips prepared with mPEG2000. Analytical parameters calculated using equations 2 and 3 on data reported in A or B (C). The PEG SAM is incubated overnight. Data are reported as mean value of three different chips and error bars represent the standard deviation.

The opportunity of adding Tween 80 in the buffer was also evaluated, since it might interfere with the measurements of anti-PEG antibodies due to its similarity to PEG [35]. The results (Figure S2) confirmed that Tween 80 had a slight inhibitory effect on the recognition of anti-PEG IgG, in running buffer or diluted HS, and therefore, for the subsequent experiments a buffer without Tween 80 was used.

Chip Regeneration

The possibility to regenerate the sensor surface is important for increasing the number of measurements and confirming the robustness of the system. However, regeneration solutions can compromise the biofunctional layer reducing the possibility to re-use the sensor. To find the proper

solution among different cocktails of commercial stock regenerating solutions (as indicated in the Supplementary file), SPR measurements were initially performed with a standard instrument, which allows to directly visualize the binding kinetics. The solution based on equal volumes of DMSO, formamide, ethanol, acetonitrile and 1-butanol, 20mM EDTA and water (UCw) resulted the most efficient for the complete removal of bound anti-PEG (IgG) and anti-PEG (IgM) antibodies without affecting the binding properties of the immobilized PEG.

The same solution was therefore tested on the SPR-POF optical chip by performing a static incubation of the UCw solution for 2 minutes, followed by 10 washing steps in MilliQ water before performing a new antibody incubation. The solution confirmed efficient in antibody removal, while maintaining the sensor ability to recognize the anti-PEG IgG antibody up to five regeneration cycles (Figure 5A). However, even if the wavelength shifts in response to the antibody binding remain, after each regeneration step, almost the same (Figure 5A), a general decrease of the absolute wavelengths corresponding to the spectrum minima occurs, possibly caused by the infiltration of the solvents contained in the regeneration solution under the gold layer (Figure 5B).

A B

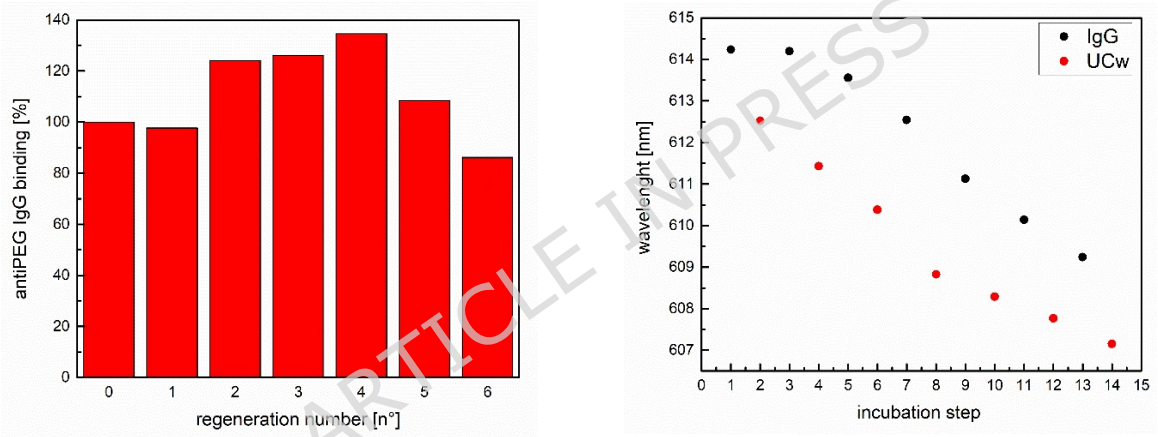


Figure 5*:* Anti-PEG IgG binding percentage on SPR-POF chip using UCw regeneration solution (A) and wavelength shift after the regeneration steps and the subsequent IgG binding (B).

Comparison with standard techniques

While the method was characterized using commercially available anti-PEG antibodies, the innate biodiversity of antibodies generated in human subjects [36] required the analysis of real samples, to verify the ability of the system to efficiently detect human anti-PEG antibodies. For this, we analyzed plasma obtained from three healthy subjects, three hemophilia A patients exposed to PEGylated FVIII, and three hemophilia A patients never exposed to PEGylated FVIII. Rather than for a clinical significance, samples were selected on the basis of results obtained with both ELISA and standard SPR analysis: three plasmas found positive for anti-PEG with both ELISA and SPR (positive controls), three plasmas found negative with both ELISA and SPR (negative controls) and three plasmas found positive with ELISA but not SPR (to check for sensitivity). The plasma samples were diluted 10 times in TSB buffer (75mM Tris-HCl, 450mM NaCl, 5mM EDTA, 0.1% w/v BSA at pH 7).

Commercial anti-PEG antibodies used for the setup of the SPR assay, i.e., IgGs from Abcam, have very slow dissociation rates from immobilized PEG, as assessed with standard SPR assay (Figure S3); consistently, their recognition is not affected by the number of washes (Figure S4 shows that after 3 washings, up to 9 additional washings steps had no further effects).

However, to take into account the possibility that the anti-PEG antibodies might have a fast dissociation constant (e.g., commercial IgGs from Acrobiosystem, purple lines in Figure S3, as well as patient's anti-PEG antibodies, Figure S5), the number of washing steps was reduced to 5 to avoid losing rapidly-dissociating antibodies.

The results (Figure 6) show that there is a very good agreement between the two plasmonic instruments, while neither of them were able to recognize the presence of anti-PEG antibodies in three samples found positive with ELISA only, likely due to a lower sensitivity of the SPR-based assays. Notably, patient #9, showed high levels of anti-PEG antibodies with all three techniques. These antibodies are characterized by a fast dissociation rate (Figure S5).

ELISA	-	-	-	+ 42.8 nM (eq)	+ 14.7 nM (eq)	+ 4.5 nM (eq)	+ 0.5 nM (eq)	+ 83.3 nM (eq)	+ 74.4 nM (eq)
SPR	-	-	-	-	-	-	+ 4 nM (eq.)	+ 15 nM (eq.)	+ 90 nM (eq.)

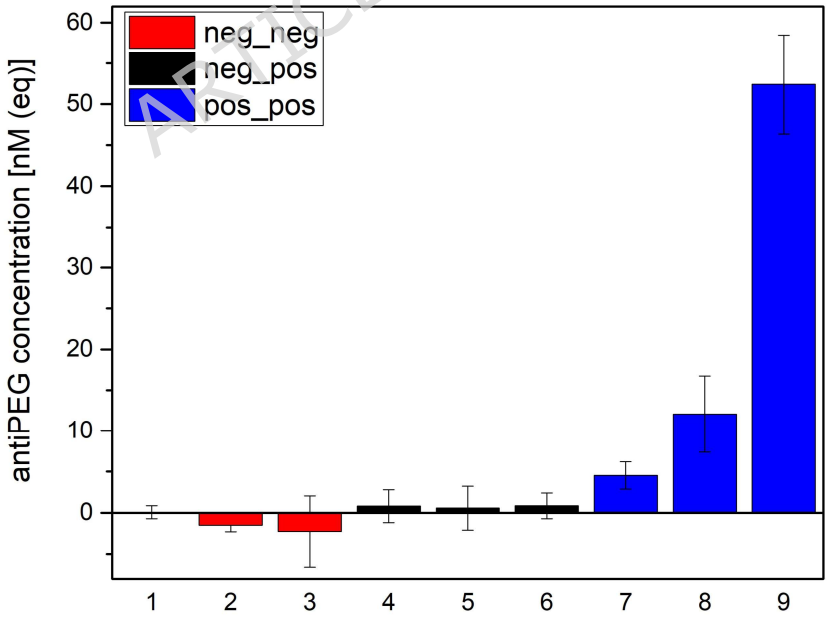


Figure 6: Summary of the anti-PEG antibodies concentration based on IgG calibration curve (reported in Figure 4A, 10x diluted HS) in all 9 samples as measured by SPR-POF instrument. Each bar is the mean value of four measurements for each sample, while error bars represent the

standard error. The results obtained by ELISA and SPR in the same samples are shown above. Plasma from: 1,5,8 healthy subjects; 3,4,9 hemophilia A patients treated with PEGylated FVIII; 2, 6, 7 hemophilia A patients never treated with PEGylated FVIII. The concentrations of anti-PEG antibodies determined with the SPR and ELISA assays are expressed as nM Equivalents, to highlight that the antibodies used for the calibration curves (IgG from Abcam for SPR and a chimeric IgG1 from CD Creative Diagnostic for ELISA) are different from those produced by the patients.

While it is true that the sensitivity of ELISA is higher than that of the two SPR-based assays (as observable by the values reported in the upper panel of Figure 6), this does not necessarily imply a limitation of the latter; the cut-off value associated with clinically relevant concentrations of anti-PEG antibodies remains in fact to be identified. Analysis are ongoing to evaluate with a sufficient number of patients the sensitivity and specificity of the different methods in relation with the clinical output. If SPR-based assays will prove to be sensitive enough to recognize clinically relevant anti-PEG antibodies, the SPR-POF sensing platform described in this article can be applied to analyze real patient samples offering significant advantages over the ELISA assay, such as the ability to perform label-free, real-time detection tests by applying the sample (appropriately diluted) directly to the sensing surface without the need for further processing, thereby significantly reducing the waiting time for results for urgent point-of-care testing at the patient's bedside.

Even if the developed system suggests a possible future employment in real scenarios, some limitations should be acknowledged. First, the results are obtained on a small cohort of patient samples and a higher number of samples should be used to fully validate the system and second, it was tested on hemophilia A patient samples and different samples with anti-PEG antibodies should be used to generalize the applicability of the proposed device. However, the aim of the work is to propose a solution for measuring anti-PEG antibodies in short time with a good accuracy (as suggested by the agreement with the standard techniques); fully validation and test with different patient' samples will be the subject of future works.

Conclusions

The screening and monitoring of the anti-PEG antibodies in patients' plasma just before and during a medical treatment could be of great relevance for guiding rationale clinical decisions when administering a PEGylated drug or for switching to a non-PEGylated one. In this context, the development of a simple and rapid POCT is highly desired to fulfill this demand. A POCT built in 3D-printing technology based on SPR-POF is here proposed: the low-cost optical chips are easily functionalized by immobilizing thiol-modified PEG chains directly on their gold surface and the protocol measurement is performed in only 15 minutes. The sensor modification is assessed by chemical-physical analyses. The LoDs reached are in the nanomolar range, both in buffer solution and in complex matrix (human serum diluted 30, 20 or 10 times in buffer). The chips are regenerable and the device has been tested on real human plasma samples, comparing the results

with standard SPR measurements performed with a commercial instrument and the ELISA assay, as well, showing good concordance for cases showing treatment failure. The proposed device addresses these unmet needs by enabling rapid, point-of-care detection of anti-PEG antibodies directly in the outpatient setting. This might allow clinicians to identify treatment failure in patients already receiving PEGylated therapies, as well as to screen patients prior to initiating a PEGylated product, both scenarios being currently unaddressed by routine clinical practice. By delivering actionable results at the time of the clinical encounter, the device has the potential to reduce diagnostic delays, avoid unnecessary drug administration, and possibly lower both direct and indirect costs, paving the way for real-world clinical implementation.

Materials and Methods

Materials

Methoxy-polyethylene glycol (mPEG) at different molecular weights (400, 1000, 2000, 5000, 10000 Da) are purchased from BiochemPeg (Gentaur Europe BVBA, Kampenhout, Belgium). Rabbit Monoclonal Recombinant Anti-Polyethylene glycol antibody IgG (ab170969), that recognizes the terminal methoxy group of the PEG molecule and does not cross react with non-specific targets in blood or serum, and rat monoclonal Anti-Polyethylene glycol antibody IgM (ab94764), that recognizes linear 20 kDa PEG, linear 5 kDa PEG, linear 600 Da PEG and 10 kDa branched PEG, are purchased from Abcam. Human serum (HS), bovine serum albumin (BSA), dimethylsulphoxide (DMSO), formamide, ethanol, acetonitrile, 1-butanol, Ethylenediaminetetraacetic acid (EDTA), Tween 80 and powder for buffer solutions are purchased from Sigma-Aldrich (Milan, Italy).

Flat gold substrates are also prepared, to allow a proper characterization of the functionalization procedures, by depositing 10 nm of titanium on a silicon substrate (100), followed by 100 nm of gold purchased from MicroFabSolution srl (Trento, Italy).

Surface functionalization

The flat gold substrates or the SPR plastic optical fiber chips are first cleaned using an Argon plasma 6.8 W for 2 minutes to remove organic contamination. Immediately after cleaning, 100 □L of mPEG-SH solution at 0.2 mM in MilliQ water are dropped on the surfaces or optical chips and let incubate in a humid chamber for 90 min or overnight. After incubation, the surfaces or chips are washed three times in MilliQ water.

Surface characterization

The static contact angle is measured using a home-made system, depositing 1 μL of deionized water droplets on the gold substrate (at least four drops per sample). The images are acquired with a CMOS camera and analyzed by Drop-Analysis, a plugin of Fiji software [37]. The results are reported as average value and standard deviations. XPS analysis is performed using a Kratos Axis Ultra DLD instrument (Kratos Analytical Ltd, England) equipped with a hemispherical analyzer and a monochromatic Al Kα (1486.6eV) X-ray source, in spectroscopy mode. Survey spectra and the principal core levels (O 1s, C 1s and Au 4f) are acquired at 0° emission angle and the relative elemental percentage is obtained by integrating the area under the core lines, applying the Shirley

background subtraction, and by correcting for the atomic sensitivity factors through a dedicated software [38].

The overlayer thickness (d_{OL}) was calculated based on attenuation length of the Au $4f_{7/2}$ signal using the following equation:

$$d_{OL} = \square_{OL} \ln (I^0_{Au}/I_{Au}) \quad (1)$$

where I^0_{Au} is the measured intensity of the Au $4f_{7/2}$ peak on a pristine gold sample, I_{Au} is the measured intensity of the Au $4f_{7/2}$ peak of gold sample exposed to the solution, and λ_{OL} is the attenuation length of Au $4f_{7/2}$ photoelectrons in the polymer overlayer (3.45 nm) [30].

Optical device, experimental method, and data analysis

A miniSPR instrument to perform the binding experiments is used (model Spectra 340 manufactured by Moresense Srl, Milano, in collaboration with University of Campania "L. Vanvitelli"). The experimental setup is based on a spectrometer VIS range 500–730 nm and a white led light source 400–780 nm, assembled as reported in Figure 1. The optical platform is based on SPR plastic optical fiber (POF) chips (model RA1008 manufactured by Moresense Srl, Milano, Italy) built with additive manufacturing technology.

Once functionalized, the optical chip is inserted into the miniSPR and preliminary equilibration is performed. First the transmission spectrum of the air (reference) is acquired, then MilliQ water is dropped onto the chip and the new transmission spectrum is acquired. After that, TBST or diluted HS samples are dropped onto the chip and let stabilize until the spectrum showed no changes. After this equilibration procedure that takes almost 1 h, ~~Then,~~ the measure with commercial anti-PEG antibodies spiked in buffer or in diluted serum or 10x diluted plasma samples is performed in a total time of 15 minutes: ~~an~~ the incubation is performed dropping 80 □L of solution over the chip and let act for 10 minutes. After incubation, the chip is washed 10 times with buffer solution (or 5 times for plasma samples), and a final measure (spectrum acquisition) is performed before proceeding with a new incubation.

Commercial anti-PEG IgG or IgM are spiked in a range of 1÷300 nM in TBST buffer or diluted human serum. Data reported in Figure 1 and Figure 3 are the mean of three different chips and error bars represent the standard deviation, while data reported in Figure 5 are obtained testing four different chips and are represented as mean value and standard deviation.

Data analysis was performed by normalizing the transmission spectrum of the buffer solution (sample) to that of air (reference) following the incubation and washing procedures. The resulting ratio spectrum was subsequently fitted using an asymmetric Gaussian–Lorentzian function to accurately determine the spectral minimum. A custom software package was developed to perform this analysis utilizing R, a language and environment for statistical computing and graphics [39]. Data are then plotted using Origin software.

The dose response curve is obtained by incubating different amounts of anti-PEG antibodies and a Langmuir fitting equation is termed in the following Hill equation (Equation 2) by considering n=1:

$$|\Delta\lambda_c| = |\lambda_c - \lambda_0| = |\Delta\lambda_{max}| \cdot \frac{c^n}{K^n + c^n} \quad (2)$$

where c represents the anti-PEG antibody concentration, λ_c represents the plasmonic resonance wavelength at concentration c, λ_0 is the resonance wavelength of the blank, $\Delta\lambda_{max}$ is calculated as the difference between the resonance wavelength at the saturation value and the blank. In general, the parameters n and K represent the Hill fitting constants. When n=1, as considered in this case, the Hill model coincides with the Langmuir model and $K = 1/K_{aff}$, where K_{aff} represents the affinity constant.

Equation (3) is used to calculate the LoD, as here reported:

$$LoD = \frac{3*(St.Dev.)\,\Delta\lambda_0}{\frac{(\Delta\lambda_{MAX}-\Delta\lambda_0)}{k}} \quad (3)$$

where $\Delta\lambda_0$, $\Delta\lambda_{MAX}$ and k are the parameters obtained from the fit using equation (2) and used to define the Sensitivity at low concentration as $\frac{(\Delta\lambda_{MAX}-\Delta\lambda_0)}{k}$.

Standard SPR binding experiments

For the present studies, a ProteOn XPR36 Protein Interaction Array system (Bio-Rad Laboratories) was used. This system allowed to immobilize PEG-BSA (to detect anti-PEG antibodies) and BSA (as reference surface) in parallel strips of the same sensor chip (CMD200M from Xantec), by classic amine coupling chemistry [40]. After rotation of the fluidic system [40], 30-fold diluted plasma samples (in Tris-buffered saline (TBS) with 0.1% BSA and no Tween), were flowed in parallel surfaces on both immobilized ligands for 300 secs at 30 µl/min, followed by a 10 min dissociation phase with running buffer only (TBS+0.1% BSA). All these assays were done at 25 °C. The SPR signals in the surfaces immobilizing PEG-BSA were corrected by subtracting the nonspecific response in the reference surface (BSA).

Standard ELISA assays

At the Hemophilia and Thrombosis Center of Milan, an in-house ELISA with minor modifications to the method described by Valsecchi et al. [13] was used for the simultaneous detection of IgG and IgM antibodies against three PEGylated FVIII products in plasma of healthy subjects and patients with hemophilia A. Specifically, IgM samples were diluted 1:20 rather than 1:10 as described in the original method. In addition, an in-house ELISA for the detection of anti-PEG IgG and IgM antibodies was developed and used on the same samples.

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Author contributions statement

L.P.: Conceptualization; Data curation; Formal analysis; Methodology; Writing – original draft; Writing – review & editing. N.C.: Conceptualization; Data curation; Methodology; Writing – review & editing. C.R.: Data curation; Investigation; Formal analysis; Writing – review & editing. R.C.: Conceptualization; Methodology; Writing – review & editing. L.V.: Formal analysis; Data curation; Investigation; Writing – review & editing. M.B.: Conceptualization; Data curation; Methodology; Writing – review & editing. R.G.: Conceptualization; Methodology; Validation; Writing – review & editing. L.P.R.: Data curation; Investigation; Formal analysis; Writing – review & editing. I.T.: Data curation; Investigation; Formal analysis; Writing – review & editing. L.S.: Methodology; Validation; Formal analysis; Writing – review & editing. C.V.: Conceptualization; Methodology; Validation; Writing – original draft; Writing – review & editing. C.S.: Methodology; Validation; Formal analysis; Writing – review & editing. F.P.: Conceptualization; Funding acquisition; Methodology; Project administration; Supervision; Writing – review & editing. M.G.: Conceptualization; Methodology; Data curation; Supervision; Writing – original draft; Writing – review & editing. L.Z.: Conceptualization; Funding acquisition; Methodology; Project administration; Supervision; Writing – review & editing.

Ethical statement

The study was part of a pharmacological observational study, named "Anti-PEG antibodies and their pathophysiological role in the personalized management of patients with hemophilia", funded by the Italian Minister of Health. Ethics Committee approval was obtained on 26th January 2023 (ID 3289, study number 6513) from the Comitato Etico Milano Area 2 (approval number 24_2023bis). The study was carried out in conformity with the 2013 revision of the Declaration of Helsinki and the code of Good Clinical Practice. Consecutive adult persons affected with severe/moderate hemophilia A referring at the Angelo Bianchi Bonomi Hemophilia and Thrombosis Center of Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Milano, Italy were asked to participate in the study and signed the informed consent.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information File