

Review

Surface plasmon resonance (SPR)-based biosensor technology for the quantitative characterization of protein–carotenoid interactions



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ARTICLE INFO

Article history:

Received 20 October 2014

and in revised form 22 November 2014

Available online 13 December 2014

Keywords:

Age-related macular degeneration

Biosensor

Carotenoid-binding proteins

GSTP1

IRBP

Macula

Retina

StARD3

Xanthophylls

ABSTRACT

The surface plasmon resonance (SPR) biosensor method is a highly sensitive, label-free technique to study the non-covalent interactions of biomolecules, especially protein–protein and protein–small molecule interactions. We have explored this robust biosensor platform to study the interactions of carotenoid-binding proteins and their carotenoid ligands to assess the specificity of interaction, kinetics, affinity, and stoichiometry. These characterizations are important to further study uptake and transport of carotenoids to targeted tissues such as the macula of the human eye. In this review, we present an overview of the SPR method and optimization of assay conditions, and we discuss the particular challenges in studying carotenoid–protein interactions using SPR.

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Introduction

Surface plasmon resonance (SPR)¹ biosensing is a spectroscopic technique that quantitatively measures binding events in real time without labeling the interacting molecules [1–3]. First demonstrated in 1983, it measures the refractive index changes when the molecules interact at the sensing surface. This technology is widely used in studying biomolecular interactions in pharmaceutical engineering, food sample analysis, antigen–antibody characterization, and basic science research [3–7]. Although there are many techniques available to characterize these interactions, most of them are time-consuming and require labelled reporter molecules such as fluorophores. Among the various biophysical transducers available, SPR has an advantage because of its reliable instrumentation, automation, disposable sensor chips, and versatility due to the wide variety of surface chemistries and assay methods available for various biomolecules [8].

Our laboratory has been working over a decade to characterize carotenoid-binding proteins in the human macula as a means to understand the mechanisms underlying the protective effects of lutein and zeaxanthin against various eye diseases. It is important to characterize the binding kinetics of these interactions to understand the nature of binding, stoichiometry, and other biophysical characteristics. This will provide a better understanding of the pharmacokinetics and pharmacodynamics of the carotenoid of interest. The binding information also helps to suggest suitable candidate compounds to further characterize protein–carotenoid complexes of interest using other biophysical methods such as X-ray crystallography. Apart from humans, carotenoid-binding proteins have been reported in plants, bacteria, insects, and crustaceans [9–16]. Over the years, researchers have used different biophysical characterization techniques to study the interaction of carotenoids and proteins which have included classical equilibrium binding methods [17], pull-down assays [18], and circular dichroism spectroscopy [19]. In the past, we studied carotenoid–protein interactions using an equilibrium binding method, but this was time-consuming and required considerable amounts of precious reagents. A fast and accurate binding assay that consumes lesser amounts of protein and ligands would facilitate the more thorough characterization of these proteins. Because of this need for an alternative binding technique, we turned to surface plasmon resonance-based binding assays to study protein–carotenoid binding interactions. In this review, we provide an overview of the

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¹ Abbreviations used: SPR, surface plasmon resonance; MP, macular pigments; SR-B1, scavenger receptor protein B1; ISX, intestinal transcription factor; HSA, human serum albumin; LDL, low density lipoprotein; VLDL, very low density lipoprotein; AMD, age-related macular degeneration; StARD3, steroidogenic acute regulatory domain protein 3; RPE, retinal pigment epithelium; LED, light emitting diode; RU, response unit; SML, sucrose monolaurate; NSB, non-specific binding; CMC, critical micellar concentration; GSTP1, glutathione S-transferase Pi isoform.

major proteins involved in carotenoid uptake and accumulation in the human retina, and we discuss the challenges and successes of our characterization of their binding affinities using the surface plasmon resonance biosensor technique.

Macular carotenoids

Carotenoids are tetraterpenoid pigments found in plants, bacteria, and fungi that are among the most widely distributed colored compounds in nature [20]. These pigments have a characteristic light absorption range in the visible region mainly at 400–500 nm [21]. They can be broadly classified into carotenes (hydrocarbons without oxygen) and xanthophylls (which contain oxygen). In plants, these pigments help absorb light energy for photosynthesis, and they protect the chloroplasts from oxidative damage [22]. These pigments also play an important role as antioxidants, protecting against peroxidative reactions that are mediated by photosensitization [23,24]. In the human eye, xanthophylls are concentrated in the macular region of the retina. Epidemiological studies suggest that low levels of macular pigments (MP) are related to the risk of developing macular degeneration (AMD) [25–29]. It is believed that MP protects against cellular damage caused by reactive oxygen species. MP carotenoids can absorb short wavelength visible light and may thus protect the retinal photoreceptor cells from damage. This is particularly important for people with intraocular lenses after cataract surgery where the protection from natural crystalline lens is no longer available [25]. Although there are over 600 carotenoids in nature, only a small percentage is known to provide beneficial effects in humans [20]. In a typical human diet, we consume over 60 carotenoids; however, only 10–15 different carotenoids actually enter the serum [30,31]. This means that selectivity already occurs at the first level of uptake in the gut. After absorption from the diet, carotenoids are concentrated in some tissues in a non-selective manner, but in the human macula the uptake process is highly selective for just two specific dietary carotenoids, lutein and zeaxanthin. In nature, such a high degree of selectivity is typically mediated by high affinity binding proteins.

Uptake and transport of macular carotenoids

Carotenoids from the ingested food are first taken up by the intestinal mucosal cells after saponification of ester linkages to fatty acids (if necessary) and lipid micellization [32,33]. In vitro studies with caco-2 intestinal cell lines and ARPE-19 retinal pigment epithelial cell lines have demonstrated an important role for scavenger receptor protein B1 (SR-B1) in the selective uptake of carotenoids into the gut and into the eye [34]. Along with SR-B1, an intestinal transcription factor (ISX) was also found to participate in carotenoid uptake by a negative feedback regulatory mechanism [35,36]. The circulatory carotenoid carrier proteins, which include human serum albumin (HSA), high density lipoprotein (HDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL) also play important roles as relatively nonspecific carotenoid carriers to target tissues [37].

Ocular carotenoid transport and binding proteins

The fovea of the human macula appears as a distinct yellow spot. Bone and Landrum identified that the yellow color is caused by the presence of two dietary carotenoids, (3R,3'R,6'R)-lutein, (3R,3'R)-zeaxanthin and a non-dietary metabolite (3R,3'S-meso)-zeaxanthin [38,39]. They further concluded that these three carotenoids, also known as macular pigments (MP), are present at the fovea in a 1:1:1 ratio. Fig. 1 shows the macula and the structures

of the major carotenoids of the human retina. It has been suggested that MP enhances visual acuity and protects against light-induced oxidative damage. HPLC analysis of the donor eyes with age-related macular degeneration (AMD) revealed that MP was ~30% lower than the levels found in age-matched control eyes, consistent with its putative role as a scavenger for reactive oxygen species produced in retinal cells [40]. The major specific carotenoid-binding proteins in the human macula have been identified as the pi isoform of glutathione S-transferase (GSTP1) for zeaxanthin and steroidogenic acute regulatory domain protein 3 (StARD3) for lutein [17,37]. Fig. 2 shows possible transport of pigments between retinal pigment epithelium and retinal cells.

The uptake and transport of pigments in the human retina is a complex and multistep process, facilitated by transport and binding proteins. At this time, it is still not certain whether carotenoids are primarily taken up from the choroidal or retinal vasculature, but recent studies point to a possible physiological pathway involving the proteins SR-B1 and IRBP from the choroidal circulation to the photoreceptor cells via the retinal pigment epithelium (RPE), a cell known to contain a wide variety of carotenoids [34]. An in vitro study using the ARPE-19 cell line demonstrated that xanthophyll uptake is mediated by SR-B1 which acts as a receptor on the cell surface that preferentially takes up xanthophylls [34]. This was clearly demonstrated when carotenoid uptake was significantly lowered when small interfering RNA and antibodies against SR-B1 were used to down regulate the activity of this receptor [34]. IRBP is a prominent protein of the interphotoreceptor space that is believed to facilitate the transport of 11-*cis*-retinal, 11-*cis*-retinol and all-*trans*-retinol between the RPE and the photoreceptors, a process critical for the proper function of the visual cycle [41]. IRBP also binds a variety of other physiologically hydrophobic molecules such as fatty acids [41]. Our in vitro studies using SPR showed that IRBP can also bind carotenoids with a K_D similar to that of retinoids, and we proposed that IRBP might play a similar role as a carotenoid transporter across the interphotoreceptor matrix [42]. Once lutein and zeaxanthin arrive at the retina, they can then bind with their respective binding proteins, StARD3 and GSTP1, to form the physiologically stable macular pigment.

Principles of surface plasmon resonance binding assays

SPR is an optical phenomenon that is used to detect the refractive index changes close to the sensing surface and its detector. The optical system is designed in a Kretschmann configuration that is shown in Fig. 3 [43,44]. It consists of a glass prism with a high quality optical surface, light emitting diode (LED), and a photodiode array (Fig. 3). Monochromatic light is reflected from the sensing surface at an angle (called the SPR angle) at which the reflected light is reduced to its minimal level. Energy transfer between the light and the electrons on the gold-coated sensing surface excites the metallic surface and generates oscillating electrons called plasmons. The electrical field in the plasmon waves is affected by the molecules bound on the sensing surface. When the molecular interaction takes place at the surface, the refractive index close to the sensing surface changes, which in turn will change the SPR angle of the reflected light because of the changes in the plasmonic waves. This change in SPR angle is detected by a photodiode array and the signal is expressed as a response unit (RU) which is directly proportional to the total mass of the bound ligands and is typically equivalent to 1 picogram per square millimeter. The change in RU versus time is called a sensorgram.

Ligand immobilization methods on SPR chips

The biorecognition element of SPR is the sensor chip which is usually a gold surface functionalized with suitable surface

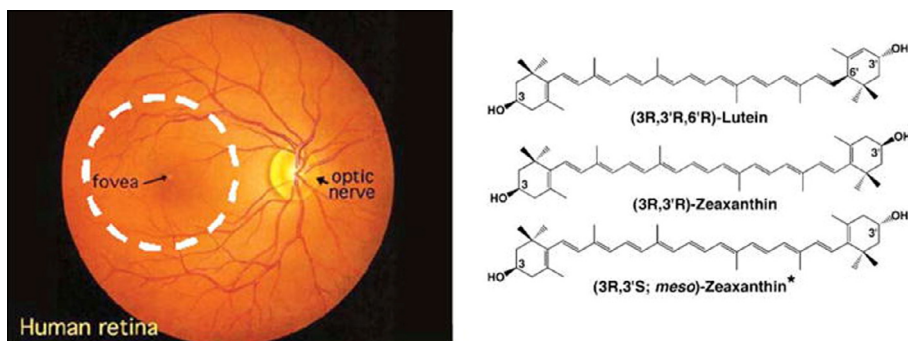


Fig. 1. Ophthalmoscopic view of a human retina (left) showing the boundaries of the human macula as a 5 mm diameter dashed white circle centered on the fovea. The macular carotenoid pigment is concentrated in the central 500 microns of the macula at the fovea. The chemical structures of the major macular pigment carotenoids are shown on the right. Reproduced from Ref. [37] with permission from *Photochemistry and Photobiology*, Royal Chemical Society.

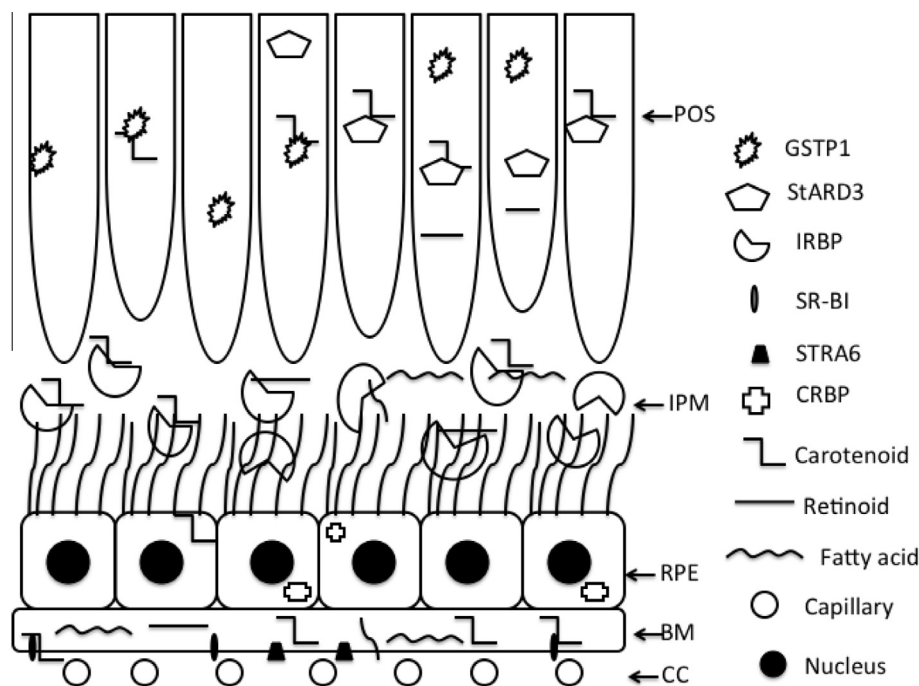


Fig. 2. Possible transport of pigments between RPE and retinal cells in mammalian visual cycle. Choriocapillaris (CC); Bruch's membrane (BM); retinal pigment epithelium (RPE); interphotoreceptor matrix (IPM); photoreceptor outer segment (POS). Reproduced from Ref. [42] with permission from *Archives of Biochemistry and Biophysics*, Elsevier.

chemistry to attach a protein or other chemical compound. In a typical SPR experiment, there are two interacting partners involved; one that gets immobilized on the sensor chip is called the *ligand*, and the second that flows through the chip surface called the *analyte*. The buffer that flows through the sensor surface is known as the *running buffer*.

The selection of appropriate binding chemistry is important to obtain high-quality data, and the versatile selections of binding chemistry immobilize a broad spectrum of ligands of interest for SPR analysis [45]. Ligands can be immobilized on the sensor surface by direct immobilization by covalent derivatization such as amine coupling, thiol or aldehyde modification. The direct coupling method requires a highly pure ligand molecule (>95%). In amine coupling, the carboxyl groups of the sensor surface are functionalized with NHS/EDC (0.05 M N-hydroxysuccinimide/0.2 M N-ethyl-N'-(dimethylaminopropyl) carbodiimide) solution to form N-hydroxy succinimide esters. These will react with the N-terminus of the ϵ -amino group of lysine to form covalent linkages with the protein of interest. Unreacted NHS esters are deactivated by 1 M ethanolamine [46,47]. For proteins with free

thiol groups, thiol coupling chemistry can be used. First, the sensor surface is activated as in the amine coupling method. It is followed by the introduction of reactive disulfide groups on the sensor by PDEA (8-mM 2-(2-pyridinyldithio)ethaneamine hydrochloride). When the protein is injected, the reactive disulfide groups will spontaneously interact with thiols on the ligands to form a covalent linkage. The deactivation of unreacted disulfide groups is carried out by injecting 50 mM L-cysteine and 1 M NaCl in formate buffer pH 4.3 [46]. Aldehyde coupling can be used to couple oxidized glycoproteins on the sensor surface. First, the sensor surface is modified with EDC/NHS. The hydrazide groups are introduced by 5 mM hydrazine in water. Unreactive esters are removed by ethanolamine (1 M). The reactive hydrazide groups will spontaneously form covalent bonds when they come in contact with the proteins containing an aldehyde group. A second approach is indirect coupling using *capture molecules* against the ligand of interest. Capture molecules can be monoclonal antibodies against the ligand, avidin to bind biotinylated protein, or antibodies to bind recombinant protein tags such as GST or histidine (His-tag). In the affinity capture method, fewer pure ligand molecules are required for

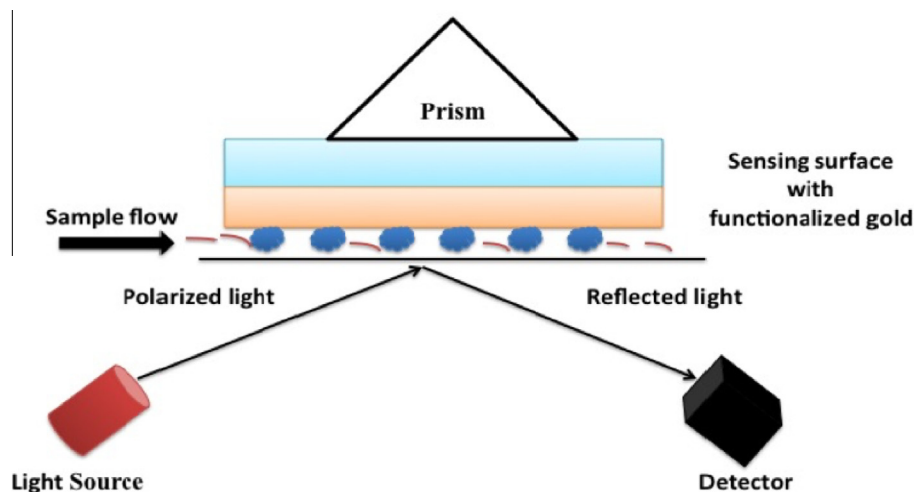


Fig. 3. The optical configuration used in SPR set up (Kretschmann configuration). The ligand is attached to the sensing surface. When it interacts with the analyte, the light source hitting the sensing surface generates spectral changes due to an angular shift.

immobilization as it is highly selective. Membrane-bound proteins can be immobilized using the liposome capture method. In this method, liposomes containing membrane-bound proteins are injected over a sensor surface coated with alkane groups (octadecanethiol) to capture those proteins [46,48].

In a typical SPR analysis, an analyte of interest passes over the ligand-bound sensor surface at a flow-rate of 10–100 $\mu\text{l}/\text{min}$. Different analyte concentration ranges are prepared in serial dilutions that cover the equilibrium binding range of the interaction. Instruments such as the SensiQ Pioneer (SensiQ technologies, Inc., Oklahoma City, OK) are equipped with newer injection techniques such as the FastStep™ and the OneStep™ methods. FastStep™ utilizes an *in situ* dilution method which creates automatic serial dilutions without the need for regeneration between each dilution step. Fig. 4 shows the schematics of the FastStep™ technique. This method is much faster than the conventional fixed concentration injections, and because the dilution series are prepared automatically, it eliminates possible human errors [49]. OneStep™ creates a continuous concentration gradient by mixing a sample and the running buffer to create a Taylor dispersion flow [50]. This method technically gives an infinite number of single concentration injections. These novel methods are reliable alternatives to conventional fixed concentration injections with dramatic improvement in analyte throughput while simplifying assay set up.

Carotenoid–protein interaction analysis using SPR

In 2004, our lab identified GSTP1 as the zeaxanthin-binding protein in the human macula [17]. At that time, binding studies were performed by incubating purified carotenoid binding protein with zeaxanthin overnight, and after equilibrium binding, unbound carotenoids were removed by three cycles of organic extraction. The bound zeaxanthin was then quantified by HPLC. The whole process took a few days and required high amounts of precious protein materials. When we identified StARD3 as the lutein binding protein, we introduced an SPR-based assay method [51]. StARD proteins were immobilized using standard amine coupling methods on the sensor surface. Then, each of the five carotenoids was dissolved in DMSO to achieve a high concentration, 10 mM PBS (pH 7.4) with 0.01% sucrose monolaurate (SML) was used as a detergent solution to solubilize carotenoids without denaturing the proteins, and 3% DMSO was employed as the running buffer. Buffer blanks were interspersed throughout the analysis for double-referencing

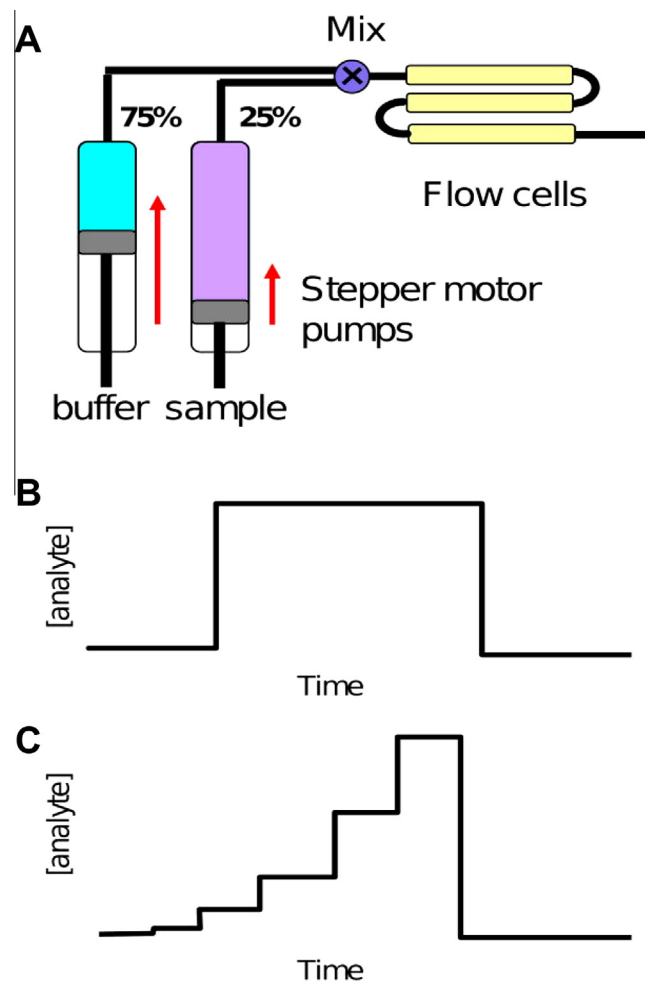


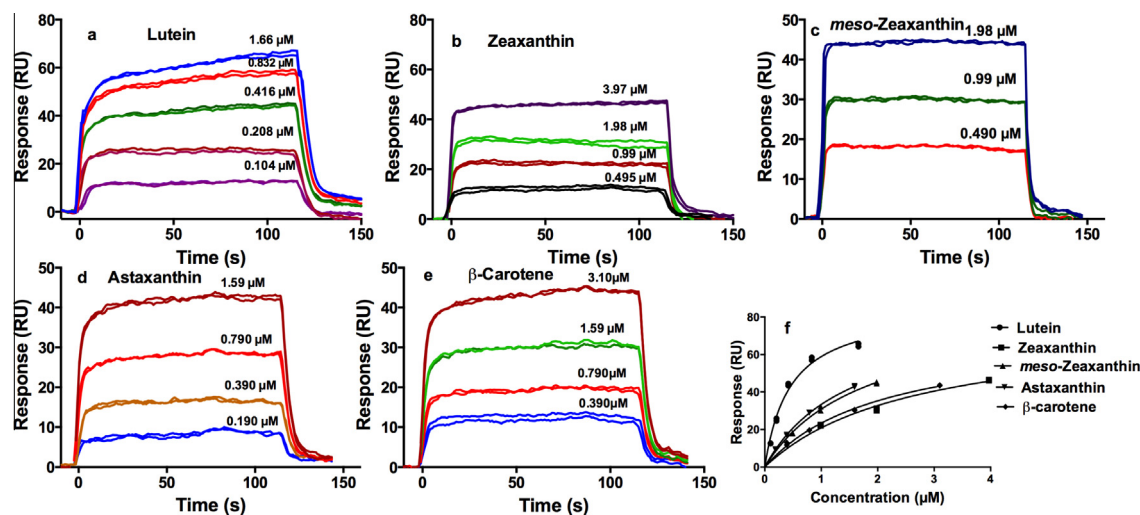
Fig. 4. Cartoon of SensiQ Pioneer flow system. Panel (A) shows two stepper motor pumps control syringes that can simultaneously deliver samples to a mixing compartment immediately prior to the set of three flow cells. Panels (B) and (C) are examples of analyte concentration profiles for standard and FastStep™. Reproduced from Ref. [28] with permission from Analytical Biochemistry, Elsevier.

purposes [52]. Fig. 5 shows the sensorgrams of the binding interactions and their respective equilibrium dissociation constants.

A major challenge in these SPR carotenoid binding assays is the hydrophobic nature of carotenoids. Common SPR surfaces such as carboxymethyl dextran can generate high levels of carotenoid non-specific binding (NSB) in the reference channels; therefore, a hydrophilic surface is needed to help reduce non-specific binding. In our initial optimization process, we evaluated various surface chemistries, and we found that polycarboxylate hydrogel seemed to reduce NSB binding optimally. Also, the assay buffer should contain suitable detergents to solubilize the carotenoids. The selection of appropriate detergent is important because it should not affect the activity of the ligand by denaturing it, and the concentration should be below the critical micellar concentration (CMC) of the detergent. If the concentration is above the CMC, the detergent will form micelles. Since the carotenoids are hydrophobic compounds, they will form lipophilic complexes with the detergents and tend to stay inside the detergent micelle without getting solubilized. The detergent also helps to prevent carotenoids from forming large carotenoid aggregates in aqueous solution. Among the various detergents screened, SML, Triton-X-100, and Tween-20 were the best behaved detergents on the SPR sensor surfaces that we used.

In 2012, we published an article on carotenoid binding studies using the next-generation SPR technique called FastStep™ [53]. It uses a novel injection method, in which carotenoid concentration series were injected as a series of seven twofold dilutions in the running buffer using the FastStep™ gradient injection mode. The results obtained were compared with the conventional fixed concentration method for accuracy [53]. We studied carotenoid-binding interactions of five proteins: human serum albumin (HSA), β -lactoglobulin (LG), two steroidogenic acute regulatory domain proteins (StARD1, StARD3), and glutathione S-transferase Pi isoform (GSTP1). HSA and LG showed relatively weak and

nonspecific interactions with the carotenoids tested ($K_D > 1 \mu\text{M}$). As shown in Fig. 6, GSTP1 exhibited high affinity and specificity toward zeaxanthin and *meso*-zeaxanthin with K_D values of $0.14 \pm 0.02 \mu\text{M}$ and $0.17 \pm 0.02 \mu\text{M}$, respectively. StARD3 was reported to have a relatively high affinity and specificity toward lutein with a K_D value of $0.59 \pm 0.03 \mu\text{M}$, whereas StARD1 showed a relatively low selectivity and affinity ($K_D > 1 \mu\text{M}$) toward the various carotenoids tested [53]. We also studied the binding interactions of interphotoreceptor retinoid-binding protein (IRBP) with retinoids, fatty acids, and carotenoids by SPR [42]. IRBP showed similar affinity toward retinoids and carotenoids ($1\text{--}2 \mu\text{M}$), while fatty acids had approximately 10 times less affinity [42]. These results suggest a role of IRBP in binding and transport of lutein and zeaxanthin in the interphotoreceptor matrix as a possible pathway for delivery of these molecules to the retina via the RPE. We recently reported that inactivity of human BCO2 as a xanthophyll cleavage enzyme relative to the robust activity of mouse BCO2 could be a possible reason for the unique accumulation of macular pigments in the primate eye [54]. SPR was used to compare the binding affinities of human and mouse BCO2 toward lutein, zeaxanthin, and *meso*-zeaxanthin. It was found that human BCO2 is 10- to 40-fold weaker than mouse BCO2 toward macular pigment binding, indicating that inefficient capture of carotenoids by human BCO2 may explain its poor cleavage activity with xanthophyll carotenoids [54]. In an accompanying article in this special issue, we evaluated the binding capacity of a complex carbohydrate, arabinogalactan, with zeaxanthin using SPR [55]. This binding study was performed to understand the kinetics of carotenoid complexation by arabinogalactan as a possible means to promote dietary uptake of carotenoids in laboratory animals and in humans. This further expands the scope of carotenoid SPR studies beyond just protein–carotenoid interactions.



Steady State Equilibrium Dissociation Constants (K_D) at 25°C

Carotenoids	Binding protein
	StARD3
Zeaxanthin	$2.60 \pm 0.10 \mu\text{M}$
<i>meso</i> -Zeaxanthin	$1.80 \pm 0.60 \mu\text{M}$
Astaxanthin	$1.54 \pm 0.02 \mu\text{M}$
Lutein	$0.45 \pm 0.01 \mu\text{M}$
β -Carotene	$2.09 \pm 0.04 \mu\text{M}$

Fig. 5. Surface plasmon resonance (SPR) sensorgrams of five carotenoids (panels a–e) binding to immobilized StARD3 (lutein-binding protein). Binding isotherm fit (panel f) of the responses shown in (panels a–e). Equilibrium dissociation constants are listed in the Table [51].

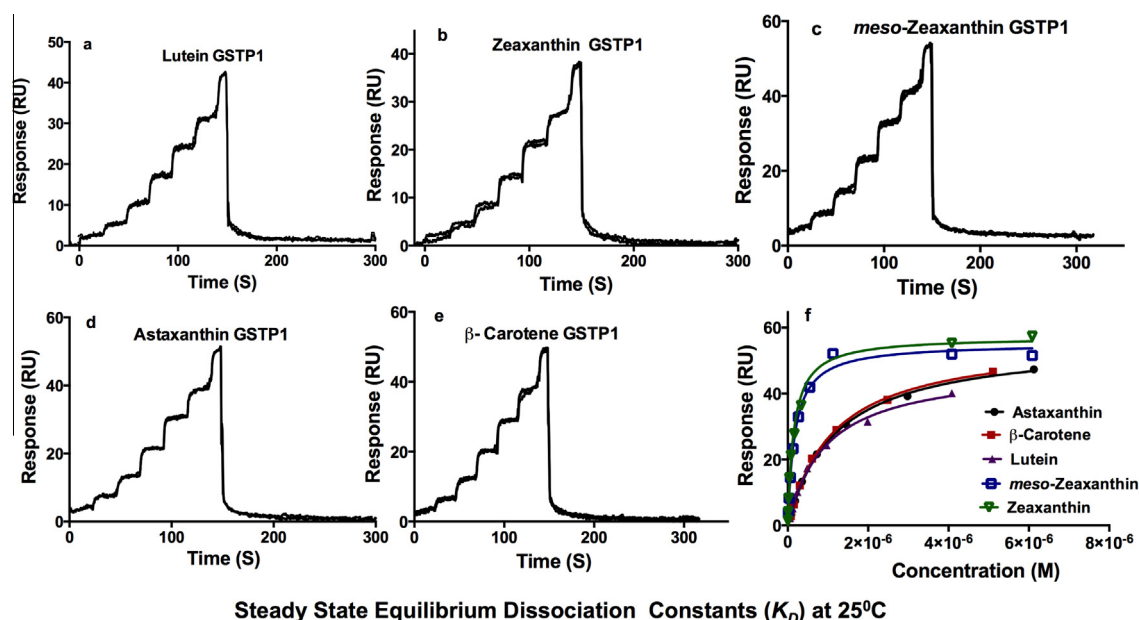


Fig. 6. Sensorgrams of five different carotenoids (panels a–e) binding to GSTP1 (Zeaxanthin binding protein) using FastStep™ SPR assay, equilibrium binding isotherm plot (f) of the responses shown in (panels a–e). Equilibrium dissociation constants are listed in the Table [53].

Conclusions

Surface plasmon resonance-based biosensors can be employed to study the interactions between physiologically important carotenoids and their binding proteins because it is simple and flexible in helping to extract binding data rapidly and reproducibly. SPR studies, followed by structural characterization of the bound complex, can help to understand the binding mechanisms. Since SPR is a non-destructive method, it helps to minimize sample usage, as the same surface can be reused for multiple analytes. We have successfully used SPR-based biosensors to study and characterize various carotenoid binding proteins related to the uptake and transport of carotenoids into the human retina, and we are beginning to examine other macromolecules that bind with carotenoids. SPR is a fast and robust technique to characterize the functional roles of these fascinating proteins.

Acknowledgments

This work was supported by NIH grant R01 EY11600 (P.S.B.) and core grant EY14800, Lowy Medical Research Foundation (P.S.B.), and Unrestricted Research Grants from Research to Prevent Blindness to the Department of Ophthalmology at Moran Eye Center, University of Utah.

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