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"Superchiral" Spectroscopy: Detection of Protein Higher Order Hierarchical Structure with Chiral Plasmonic Nanostructures

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Supporting Information

ABSTRACT: Optical spectroscopic methods do not routinely provide information on higher order hierarchical structure (tertiary/quaternary) of biological macromolecules and assemblies. This necessitates the use of time-consuming and material intensive techniques, such as protein crystallography, NMR, and electron microscopy. Here we demonstrate a spectroscopic phenomenon, superchiral polarimetry, which can rapidly characterize ligand-induced changes in protein higher order (tertiary/quaternary) structure at the picogram level, which is undetectable using conventional CD spectroscopy. This is achieved by utilizing the enhanced sensitivity of superchiral evanescent fields to mesoscale chiral structure.

Protein crystallography, NMR, and (electron) microscopy are still routinely used to elucidate higher order biological structure. These techniques, however, are incompatible with the high-throughput characterization necessary for many analytical applications, since they are both time-consuming and require significant amounts of material. Optical spectroscopic methods provide a scalable alternative, however they do not routinely provide a definitive spectral fingerprint of the higher order structure of biomolecular systems, such as the tertiary and quaternary structure of biological macromolecules and assemblies. Metamaterials are composed of nanostructures with dimensions comparable to the wavelength of light enabling them to manipulate electromagnetic (EM) fields in unique ways, affording new opportunities in optics, spectroscopic detection, and characterization of matter.[1−5] In the present study we use a new label-free paradigm in chirally sensitive spectroscopy that allows changes in the chirality of tertiary and quaternary structure of a protein to be detected at picogram sensitivity. These chiral nanostructures can be optically excited to generate chiral evanescent fields that exhibit a property called superchirality.[1,6−10] In effect superchiral fields "twist" on a shorter length scale than circularly polarized light (CPL) of the same frequency.

Proteins display chirality on a range of scales, providing potential fingerprints of protein structure over the full range of hierarchical structure. Therefore, chirally sensitive, i.e., chiroptical, spectroscopic methods based on the differential interaction of circularly polarized light are useful tools in protein structure characterization.[11,12] These methods, however, can only routinely detect secondary and, to a much more limited extent, tertiary structure content of proteins. In contrast, the superchiral evanescent near fields of chiral plasmonic metamaterials are shown here to be uniquely sensitive to both the tertiary structure and the orientation of domains within proteins (quaternary structure). These fields are more sensitive to higher order structure than CPL because there is a smaller mismatch between the effective helical pitch of a chiral evanescent field and the length scale of chirality of higher order structures, which is typically 10−100 nm. The superchiral evanescent fields display a multiscale sensitivity, which enables ligand-induced changes in higher order structure to be detected with great sensitivity. This enhanced sensitivity is in part due to the quadrupolar contribution to optical activity. Under ordinary excitation by circularly polarized light, the dipolar and quadrupolar terms can contribute to the same order of magnitude.[2] However, the superchiral fields display steep gradients, which will enhance the quadrupolar contribution relative to the dipolar contribution. Efrima[13] has discussed the influence of the gradients of localized electromagnetic fields on the quadrupolar contribution to the optical activity displayed by an adsorbed anisotropic chiral layer. Thus, superchiral polarimetry is able to detect ligand-induced changes in protein conformation.

We used a novel templated plasmonic substrate (TPS) that was created on a polycarbonate template fabricated using a high-throughput injection molding method.[14] We have fabricated template slides with indentations of nanopatterns of a "shuriken" structure which are either left or right handed (SI). The far-field chiroptical properties of the TPSs were characterized by collecting the optical rotatory dispersion (ORD) spectra in reflection mode for linearly polarized incident light. Spectra collected from left-handed (LH) and right-handed (RH) TPSs in the presence of buffer are shown in Figure 1. The spectra from the LH and RH TPSs exhibit the expected mirror image behavior with a large bisignate structure in the region from 700 to 770 nm, which dominates the spectra. Minor differences between spectra collected from LH and RH

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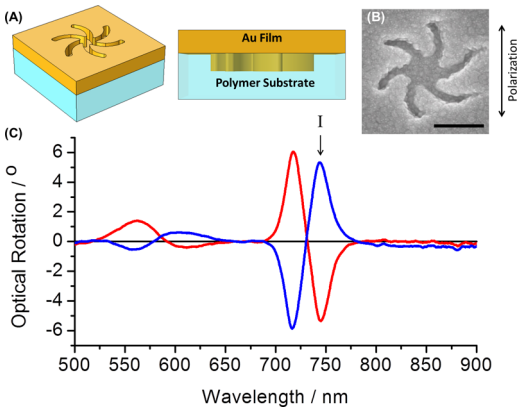

Figure 1. (A) Graphical description of a single LH nanostructure in the TPS. (B) SEM of a Shuriken nanostructure from a LH TPS (scale bar shows 250 nm). (C) ORD spectra for LH (red) and RH (blue) TPS.

substrates can be attributed to variations in the level of defects. These novel TPSs are superior to structures used in previous work[1] with 2 orders of magnitude greater optical activity and significantly lower production costs by a factor of 1000.

The shuriken structure generates evanescent fields that display-enhanced chiral asymmetry (i.e., superchiral).[1,6−10] This enhanced asymmetry is validated by our EM simulations that show that the fields around the shuriken structures display chiral asymmetry an order of magnitude greater than CPL (SI). The overall chiral asymmetry of the evanescent fields generated around a structure is governed by its handedness. Such chiral evanescent EM fields can be used to perform superchiral polarimetry, which measures the asymmetry of the effective refractive indices of chiral media on the handedness of the applied chiral evanescent field. The foundation of superchiral polarimetry employed in this study is the dependence of the spectral position of a plasmonic resonance on the dielectric environment at the surface of a nanostructure, which is the basis of the biosensing capabilities of other nanostructured plasmonic materials (SI).[1,15−18] The resonance wavelength shift ($\Delta\lambda_{L/R}$) is measured for identical chiral dielectric layers (proteins) on LH and RH substrates. The parameter $\Delta\Delta\lambda = \Delta\lambda_R - \Delta\lambda_L$ represents the asymmetry in the refractive index of the dielectric layer (protein) on the reversal of the handedness of the superchiral evanescent EM fields and is a direct analogue to the angle of optical rotation measured in a conventional polarimetry measurement. Hence achiral solutions would show no $\Delta\Delta\lambda$ values (SI). We have calculated $\Delta\lambda_{R/L}$ values using the peak labeled I in Figure 1.

We have chosen to study two well-characterized proteins from the shikimate pathway that microbes use to synthesize aromatic amino acids.[19] These two proteins undergo ligand-induced conformational changes, making them ideal test-beds for our approach. The proteins differ in size, ligand affinity, and the nature of the ligand-induced conformational change. 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) from *Escherichia coli* is a 46 kDa molecular weight protein that binds glyphosate and shikimate-3-phosphate with low micromolar level affinity. Shikimate kinase (SK) from *Erwinia chrysanthemi* is a 19 kDa molecular weight protein that binds adenosine diphosphate (ADP) and shikimic acid together with Mg^{2+} ions with high micromolar to low millimolar affinity. In both cases, protein crystallography (PX) data provide high-resolution structures of the proteins in liganded and unliganded states (Figure 2) for either the individual proteins or for proteins from related species.[20−25] The crystal structures reveal

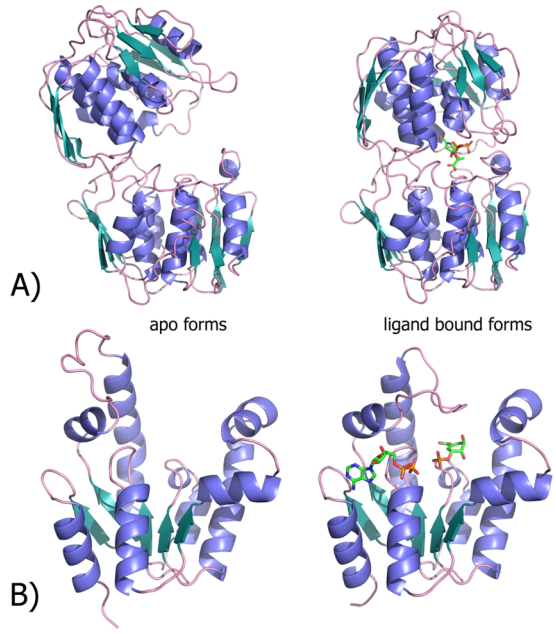

Figure 2. Ribbon representations of the crystal structures of EPSPS and SK, illustrating large domain movement and smaller loop closure on ligand binding, respectively. (A) EPSPS (PDB code 2BJB) open form (left) and the closed form (right) in the presence of ligands (PDB code 2O0E). (B) SK (PDB 2IYT) open (left) and closed (right) forms in the presence of ligands (PDB code 2IYZ). The ligands are colored according to atom type, while the secondary structure shows α-helices (blue) and β-strands (green).

that on ligand binding, EPSPS and SK undergo significant changes in domain and tertiary structure, respectively.

EPSPS exists as a monomer and has two globular domains that are connected by a flexible strand, which effectively acts as a "hinge". The ligands interact with both domains, and therefore ligand binding brings the two domains together, to produce a compact structure with a more rigid alignment of the α-helices and β-sheet. PX indicates that this change in conformation (quaternary structure) does not involve a significant change in secondary structure content. SK is a protein with a very different structure and structural changes on ligand binding. It is a small single domain structure that has a helix and loop called the "lid"-domain, which closes over the active site on ADP binding, and a helix−loop−helix motif which moves or becomes more ordered on shikimate binding. These conformational shifts induce a significant change in tertiary structure. However, comparison of the X-ray structures shows that there is likewise no significant change in the secondary structure content of SK on ligand binding.

For comparative purposes we collected UV−vis circular dichroism (CD) spectra for liganded and unliganded states of the proteins. Spectra were collected for both the far and near UV regions which are sensitive to secondary and tertiary structure respectively, (SI). In the case of SK, the near UV region is uninformative, since it overlaps a region where unbound ligand absorbs light, thus limiting the accessible spectral range of the CD spectra. A slight change is observable in the far UV, but in the limited near UV range, no significant change is observed. On its own, these data would suggest that

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there is a change in secondary structure content but no significant change in tertiary structure, which is counter to the bench mark PX data. Similar behavior of CD spectra has been observed in a related SK study.[24] The changes in the far UV region on ligand binding were attributed to an altering of the relative orientation of the α-helices and β-sheets, rather than representing a change in secondary structure content. In the case of EPSPS, neither the far nor the near UV spectra region is altered by ligand binding, indicating that in this case, CD is insensitive to changes in the quaternary structure.

Our present study demonstrates that nano plasmonic polarimetry measurements display a greater sensitivity to chirality changes caused by ligand-induced conformational changes than UV−vis CD spectroscopy. In particular, superchiral polarimetry is sensitive to the ligand-induced conformation change in EPSPS, which is undetectable to conventional CD spectroscopy.

For our studies, both the EPSPS and SK used were recombinant histidine tagged (His-tagged) proteins that were immobilized on the TPS surface using an established methodology (SI).[26] We estimate that the protein quantity adsorbed on the measured surface will be <100 pg (SI). Figure 3A,B shows ORD spectra collected from the TPS in the presence of: buffer alone, immobilized protein in buffer, and immobilized proteins in the presence of solutions of ligands. The $\Delta\Delta\lambda$ values obtained from these spectra are shown in Figure 3C. The ligand solutions contain chiral molecules: shikimic acid and ADP for SK and shikimate-3-phosphate and glyphosate for EPSPS. The anionic ligands (shikimate, shikimate-3-phosphate, glyphosate, and ADP) in the absence of proteins, produce small $\Delta\Delta\lambda$ values, but with opposite signs. The small magnitude of the $\Delta\Delta\lambda$ values (and also smaller absolute $\Delta\lambda_{L/R}$ shifts) is to be expected for small molecules which occupy a smaller volume of the available EM fields. The average shifts for the structures $[\Delta\lambda_{Av} = (\Delta\lambda_R + \Delta\lambda_L)/2]$ and dependence of $\Delta\Delta\lambda$ on surface coverage are available in SI.

Both EPSPS and SK induce positive $\Delta\lambda_{Av}$ and $\Delta\Delta\lambda$ values that are significantly larger than those induced with the ligand mixtures. When the EPSPS and SK are exposed to their ligand solutions, there are significant negative $\Delta\Delta\lambda$ values (calculated relative to the immobilized protein) observed; with the largest observed for ligand binding to EPSPS. The spacing of the protein binding sites on the TPS should permit the unhindered changes in the protein structure, as characterized by the protein X-ray structures (Figure 2). The location of the His-tag at the N-terminus of the protein means the protein molecules will be oriented in a unique and consistent way relative to the surface. Having control over the attachment of the protein to the TPS and, hence, relatively well-defined adsorption geometries facilitates interpretation of EPSPS and SK data. Superchiral polarimetry is sensitive to anisotropy in chiral structure, and this property has previously been utilized to discriminate between globular proteins with high α-helical content and more structurally anisotropic proteins with high β-sheet content.[1] In conventional chiroptical spectroscopy, the dipolar and quadrupolar terms can contribute at the same order of magnitude to the level of optical activity in anisotropic materials.[12] Alternatively, the local chiral fields around our chiral nanostructures display steep field gradients, which will enhance any quadrupolar contributions relative to the dipolar counterparts.[13] Our results clearly demonstrate that superchiral polarimetry is a very sensitive probe to changes in the

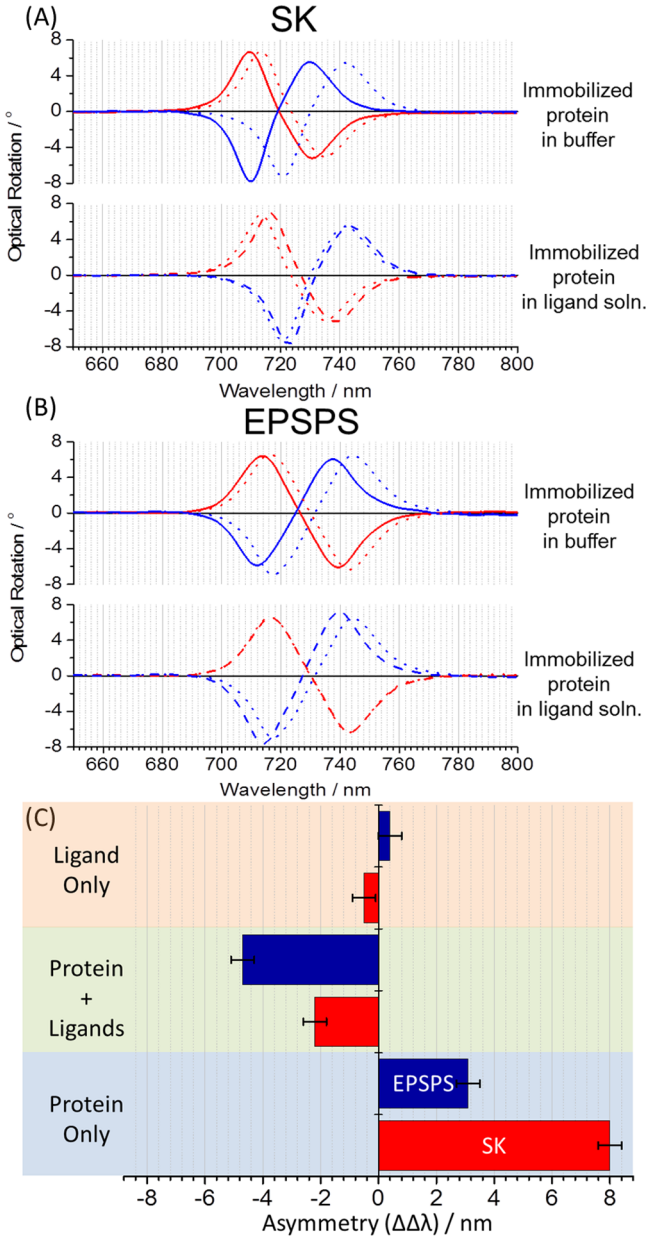

Figure 3. ORD data collected for (A) SK and (B) EPSPS. The upper plots in each panel show a shift from buffer (solid) to immobilized protein (dotted). The lower plots show shifts from immobilized proteins in buffer (dotted) to immobilized proteins in ligand solution (dashed). Note that in the EPSPS case, the LH data for immobilized proteins and ligand addition overlap. (C) $\Delta\Delta\lambda$ values for the data obtained from the ORD spectra.

anisotropic nature of protein structure induced by changes in the tertiary and domain (quaternary) structure.

In summary, we demonstrate that superchiral polarimetry can readily detect ligand-induced conformational changes in picogram quantities of proteins. This level of detection is unattainable with conventional chiroptical spectroscopic techniques, which are also inherently weakly sensitive to changes in higher order structure. Our study demonstrates the power of using evanescent fields with enhanced chirality for probing higher order structures of biomacromolecules presenting the possibility of a novel label-free biophysical measurement technology. The spectroscopic fingerprint of binding provided by superchiral polarimetry is a "static" measurement, which, in

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principle, with an appropriately engineered cell, can be performed on nanoliter quantities of ligand solution. Furthermore, given the micron-sized dimension of the nanopatterned surface required for analysis, superchiral polarimetry affords unprecedented multiplexing capabilities (≥1000 measurements) that could be performed using a single chip. Such technology could be a powerful tool in applications such a high-throughput drug screening.

■ ASSOCIATED CONTENT

Supporting Information

Experimental methods; EM simulations; superchiral polarimetry measurements method; resonance shifts by ligand only solutions; CD measurements; average shifts for experimental spectra; note on asymmetry due to proteins; protein immobilization and estimation of protein mass at surface; EPSPS surface coverage; refractive index measurement; EPSP synthase activity assay results. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b04806.

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Notes

The authors declare no competing financial interest.

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