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# Nanocrystalline diamond sensor targeted for selective CRP detection: An ATR-FTIR spectroscopy study

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**Abstract**

Protein immobilization on functionalized fluorine terminated nanocrystalline (NCD) films was studied by attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy using an immobilization protocol developed to specifically bind C-reactive protein (CRP). Using an ATR-FTIR spectroscopy method employing a force-controlled anvil type configuration, three critical steps of the ex-situ CRP immobilization were analyzed. First, the NCD surface was passivated by deposition of a co-polymer layer consisting of polyethylene oxide and polypropylene oxide. Second, a synthetic modified polypeptide binder with high affinity to CRP was covalently attached to the polymeric film. Third, CRP dissolved in aqueous buffer in concentrations of 10-20 μg/mL was added on the functionalized NCD surface. Both the amide I and II bands, due to the polypeptide binder and CRP, were clearly observed in ATR-FTIR spectra. CRP amide I bands were extracted from difference spectra, and yielded bands that agreed well with reported amide I band of free (non-bonded) CRP in solution. Thus, our results show that CRP retains its secondary structure when it is attached to the polypeptide binders. Compared to previous IR studies of CRP in solution about about 200 times lower concentration was applied in the present study.

Keywords: Infrared spectroscopy, ATR-FTIR, nanocrystalline diamond, CRP, protein binders, biosensor

## Introduction

Biosensors find applications in several different disciplines, such as medical diagnostics, environmental sensing, food industry, forensics and security [1]. In general, their function relies on a recognition event, where biomolecules, for example proteins, nucleic acids and (synthetic) polymers, specifically bind to target molecules, whereupon a physicochemical response occurs that is

transformed into an output signal that is correlated to the number of interacting counterparts. To achieve best performance of a biosensing device it is essential to anchor biomolecules on proper substrates which are compatible with the transducer and detection system and at the same time enhances its functionality. In this context, advances in nanoscience and nanotechnology have paved the way for new possibilities for biosensors research [1-3]. An example is nanocrystalline diamond (NCD), which is at focus in this study. NCD has been reported to be a promising candidate in biosensor applications since it exhibits good electrochemical properties and is straightforward to functionalize with proteins [4]. Additionally, it possesses a number of other important properties, such as biocompability, mechanical robustness, chemical and physical stability, and optical transparency in a wide wavelength regime [5]. In a recent proof-of-concept study modified NCD surfaces were characterized and functionalized to be utilized in a biosensor setup for C-reactive protein (CRP) [6]. Modified NCD surfaces were analyzed in an ELISA set-up, which in comparison with a standardized antibody based ELISA protocol using conventional polymer substrates, showed substantially higher signal-to-noise (S/N) ratio. To realize functional NCD surfaces different preparation steps were undertaken. Stepwise modification was analyzed by X-ray photoelectron spectroscopy (XPS) to ensure accuracy of the immobilization process.

In general, development of biosensors requires that each fabrication step is optimized, which is a tedious and demanding task. Ideally, it is desired to perform surface analysis of each modification step non-destructively and preferably in-situ. For such measurements label-free vibrational spectroscopy is an important analytical tool. In this study we employ attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR). To gain proper ATR-FTIR signal from a solid material it has to be in good optical contact with the internal reflecting element (IRE), which is normally done by pressing the material of interest onto the IRE. In more elaborate schemes, the IRE itself is functionalized and acts as a simulated sensing layer [7]. We have previously reported a novel method to analyze ex-situ immobilization steps of functionalized sensor chips by pressing them with a force-controlled anvil-type diamond IRE [8, 9]. In that study, discrimination between the co-polymer, peptide and target substance, an organophosphate, was established without loss of functionality. In addition, secondary peptide structure was interpreted from its amide I band.

There are several advantages to apply vibrational spectroscopy relative to other technologies. No labelling is needed, as is common in fluorescence based assays, which otherwise may induce structural changes or alter the functionality. In contrast to fluorescence and NIR spectroscopy, mid-IR (MIR) spectroscopy measures the fingerprint region, which provides chemical identity. It is important to remember that MIR spectroscopy is not mass sensitive technique, as for example those based on surface plasmon resonance (SPR) or quartz crystal microbalance (QCM). In contrast to other surface functionalization schemes, NCD can be functionalized by quite simple

techniques [4, 10]. This means that both small as larger organic compounds are detected, and if unwanted molecules interact with prepared surface they could be revealed from deviating spectral peaks (so called non-specific binding). Finally, from spectral analyses structurally information can be obtained, such as secondary structures of proteins.

C-reactive protein (CRP) is a plasma protein showing manifold elevated levels upon inflammation and infections, and, among other examples, is also considered to be a specific biomarker of heart attack [11]. Therefore it is of huge interest to have access to easy, rapid and reliable methods in clinical analysis to probe CRP concentration in real samples. CRP belongs to the group of proteins called pentraxins exhibiting five identical subunits non-covalently bound to its neighbors to form cyclic quaternary structure [12]. Twenty years ago the secondary structure of CRP, and related pentraxins, such as female protein (FP) and serum amyloid P component, were studied with IR spectroscopy. In particular, the effects on conformation induced by ions as magnesium, calcium and phosphorylcholine (to activate the complement system) were investigated [12, 13]. Transmission FTIR was used and the first experiments were done on FP dissolved in water [12]. By analyzing spectral shapes of the amide I band it was shown that β-sheet component was the dominating secondary structure. Quantitative spectral analysis of the amide I band indicated the following protein conformations: 50 % β-sheet, 13 % α-helix, 23 % β-turn and 14 % random coil. Thermal effects on denaturation of CRP have been analyzed with FTIR together with SPR spectrometry and differential scanning calorimetry [14]. Lately, Raman spectroscopy has been applied to study CRP in real human blood plasma samples for clinical purpose. Successful quantitative prediction of CRP levels was done by analyzing the data in terms of the partial least squares (PLS) method called variable selection method Interval PLS (IPLS) [15].

In the present study, we employ force-controlled ATR-FTIR methodology to study CRP, both in the unbound state and when it is selectively bound to functionalized NCD surfaces. Furthermore, different functionalization steps are characterized with-ATR-FTIR, and the fine structure of amide I bands is unravelled. To the best of our knowledge this is the first ATR-FTIR study of CRP, and provides new insights into the chemistry of the various surface functionalization steps and CRP bonding to the CRP binder.

## Materials and Methods

### Materials

All buffers, proteins, reagents, and substrates were purchased from commercial sources, with the exception of Pluronic F108-PDS, which was a generous gift from Allvivo Inc. A detailed description of the chemicals used can be found elsewhere [6]. A 4-inch silicon wafer with a 1 μm thick NCD film was

obtained from Advanced Diamond Technologies, Inc. The NCD coated Si wafer was diced into 1x1 $cm^2$ pieces.

**FTIR**

All ATR-FTIR measurements were made in a vacuum pumped FTIR spectrometer (Bruker IFS 66v/S) equipped with a broad band MCT detector. The infrared spectra were recorded between 4000 and 700 $cm^{-1}$ with 4 $cm^{-1}$ spectral resolution, and an optical aperture of 4 mm. The ATR is based on a commercial single reflection IRE diamond/ZnSe accessory (DuraSampl*IR* II™ from SensIR Ltd.) with an incident angle of light 45°. Upon ATR analysis, the immobilized NCD substrates are pressed with a pre-set torque-limited pressure against the protruding diamond IRE crystal. All measurements were conducted at room-temperature. First, all ATR-FTIR spectra were recorded with the cleaned diamond IRE surface as background. In subsequent surface functionalization steps the previous step was used as background. The measurements were conducted over 269 scans. The spectra were ATR-corrected to account for the wavelength dependence of the penetration depth. Spectra were then cut in the fingerprint region and baseline corrected.

**Fluoride termination of NCD**

In a previous study, fluorine, hydrogen and oxygen termination of the NCD surface were examined [6]. By analyzing the S 2p peak in X-ray photoelectron spectroscopy experiments of Pluronic F108-PDS coated NCD surfaces, it was found that highest packing density (coverage) was achieved with F-termination, probably due to its higher hydrophobicity. In this study, therefore F-termination was employed  Furthermore, a higher S/N ratio,lower unspecific binding in dose-response binding assays and better long term stability were obtained with F-terminated NCD. The NCD coated silicon substrates were cleaned in hot $H_2SO_4/H_2O_2$ (piranha solution). The fluorine termination was done in a plasma system built for reactive ion etching (SLR ICP, Plasma-Therm, USA). The process parameters were as follows. Gas flow: 10 sccm Ar and 10 sccm $SF_6$; Pressure: 50 mTorr; RF power: 50 W; and process time: 2 min. The measured contact angle (CA) was well above 100° on all fluorinated surfaces, proving the hydrophobic character of the fluorinated NCD surface.

**CRP binder**

In this study, a specially designed polypeptide with high affinity to CRP was used. The modified polypeptide consists of 42 residues organized in a helix-loop-helix fashion. Details of design and synthesis of the peptide binder can be found elsewhere [6]. Notably, the synthetic peptide is further optimized to selectively bind to CRP by conjugation with a derivative of phosphocholine, an endogenous ligand which is known to bind the CRP. It was here conjugated to the side chain of a lysine residue in binder molecules. The resulting binder is named 3-D10L17-PC6, and due to

cooperative effects it possesses enhanced affinity to CRP (dissociation constant in low nanomolar range) [16]. Furthermore, a cystein residue orthogonally protected by the acetamidomethyl (Acm) group was positioned in the loop region (amino acid residue position 24) of the polypeptide to facilitate binding to the Pluronic coated NCD surface [6, 16].

### Binding assays – immobilization steps

Pluronic F108-PDS is a co-polymer that generally binds strongly to hydrophobic surfaces. Pluronic forms a monolayer at the surface which allows specific binding of functional molecules. The fluorine terminated NCD substrates were coated with Pluronics over night in room temperature. Prior to Pluronic deposition, the substrates were washed three times by rinsing the samples with 1.5 mL milli-Q water, and once with PBS buffer containing EDTA, followed by drying the surface with a gentle flow of nitrogen gas.

The peptide CRP binder was conjugated to the Pluronic F108-PDS-coated NCD surface as described in details previously [6]. The CRP samples were prepared by adding exact amounts of purified human CRP from the same stock solution (3.5 mg/mL) to human CRP-free serum and dilute in HBS buffer containing 0.05 % bovine serum albumin (BSA) to 10 and 20 μg/mL. An incubation time of 1.5 h was used, whereupon the substrates were rinsed four times with HEPES-Tween mixture, and finally with milli-Q water to remove excess salt.

## Results and Discussion

Following the immobilization protocol developed by Fromell et al. [6], F-termination of NCD was used to make the NCD surface hydrophobic with CA > 100°. Pluronic is a tri-block copolymer consisting of a central hydrophobic part that binds non-covalently to the hydrophobic NCD surface with two hydrophilic arms directed outwards in aqueous surroundings. According to previous work the thickness of this polymeric film is expected to be about only a few nm [6]. Anchoring of the CRP binder to the Pluronic is accomplished by covalent bonding of the polypeptides' cysteine groups, which are positioned at the loop region, to the hydrophilic parts of the copolymer possessing S-S bonds. The HS group of cysteine attacks this disulfide bridge whereupon a new S-S binding is formed that is linked to the modified peptide. Finally, as a third immobilization step, the prepared NCD surfaces were exposed to a solution of CRP in Hepes buffer which results in specific CRP bonding to the phosphocoline unit of the binder.

Each of the immobilization steps described above was systematically and non-destructively analyzed with ATR-FTIR spectroscopy. Between each functionalization step the surfaces were thoroughly washed to remove unspecific bounded substances. Several NCD surfaces were prepared and

analyzed, and various spots on each NCD surface were studied. Herein representative data from these analyses are shown.

The Pluronic coated NCD surfaces exhibit only weak vibrational bands in the ATR-FTIR spectra in the finger print region (Fig. 1), as expected due to the thin layer thickness of only a few nm. Notably, the polymeric NCD coating withstands several cycle of pressing onto the diamond ATR-crystal, as verified by the reproducible ATR-FTIR spectra. Thereafter, the peptide binder immobilization step was examined in the same way. Significant peaks arise due to amide I and II bands of the binder (Fig. 1), centered at 1652 and 1545 $cm^{-1}$, respectively, in good agreement with reported ATR-FTIR spectra of related synthetic peptides ex-situ immobilized on functionalized surfaces [8, 9]. It is likely that the binder binds covalently, since the same spectral pattern was reproducibly obtained after careful washing of the substrates. Finally, ATR-FTIR measurements were performed on the same chip after CRP incubation and rinsing, resulting in a broadening of the amide I and II bands as is shown in Fig. 1 (blue spectrum). For the amide I band this is most pronounced at the lower frequency range around 1650 $cm^{-1}$, with a pronounced shoulder located at 1632 $cm^{-1}$. We ascribe this to be an effect of successful covalently binding of CRP to the binder 3-D10L17-PC6 attached to Pluronic on the NCD surface. The same effect can be seen for the amide II band, where a shoulder at around 1520 $cm^{-1}$ develops upon CRP bonding. We note that the penetration depth of the evanescent wave is estimated to be about 1.5 μm by using parameters given in related work utilizing similar experimental equipment [8], meaning that all peptide units bound in the film are probed by the electromagnetic evanescent wave. Moreover, with the ATR-FTIR technique employed here, the top-most layer is in contact with the IRE, in contrast to other approaches where the IRE itself is functionalized [7, 17], thus resulting in higher signal from the top-most sensor layer.

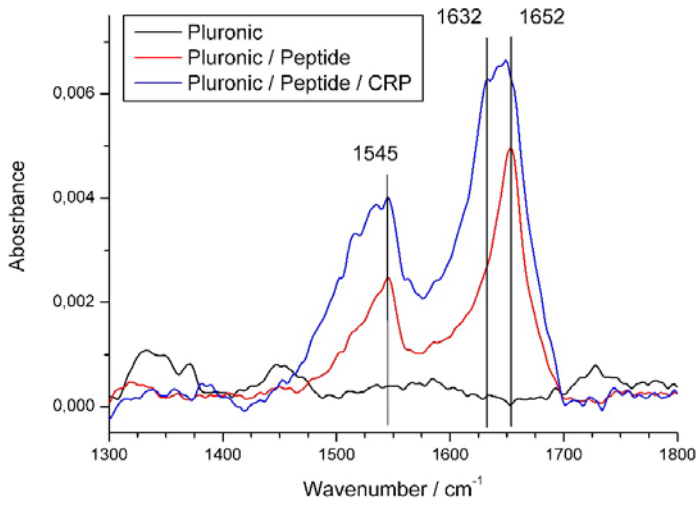


*Fig. 1 ATR-FTIR spectra measured on same NCD surface after three ex-situ sequential immobilization steps: 1) copolymer Pluronic (black), 2) added modified polypeptide binder (red), and 3) surface bound CRP (blue). Here the same Pluronic spectrum was used as background in the measurements of binder and CRP samples. The concentration of the peptide binder and CRP was both 20 μg/mL.*

A common problem when performing MIR spectroscopy of amide I bands of proteins in aqueous solutions is the strong interference of the water δ(HOH) mode. The shoulder at 1632 $cm^{-1}$ in the CRP spectrum may therefore be a consequence of that, i.e. more water is accumulated into the film.

Due to hydrophobic property of the polymer film only limited amounts of $H_2O$ is accumulated into the film in our case, and consequently only a weak MIR absorbance of ν(OH) around 3300 $cm^{-1}$ due to $H_2O$ is seen (data not shown), i.e. it is much weaker compared with those obtained with polymer films of more hydrophilic character [8]. Moreover, the absorbance of the 3300 $cm^{-1}$ band due to the binder is approximately the same with and without CRP linked . Besides, upon immobilization of the binder and CRP, respectively, both the amide I and amide II bands are resolved and show an intensity ratio that is commonly reported in literature [[8]., Therefore we disregard the possibility that the the 1632 $cm^{-1}$ is due to water, and attribute the 1632 $cm^{-1}$ shoulder to the CRP amide I band. Often when performing transmission IR of proteins, $H_2O$ is exchanged with $D_2O$ based buffer solution, leaving both amide I and II isolated without $H_2O$ interferences. This approach was adopted by Dong et al. when studying the effects of calcium, magnesium and phosphorylcholine on secondary structure of CRP [13]. In their study, the CRP concentration was 5 mg/mL, which is 200-500 times higher than employed here on water based samples. We ascribe our ability to reduce the effect of water and reaching low CRP concentration to the hydrophobic coated NCD surfaces, which effectively removes excessive water, which greatly facilitates background correction. From a technical viewpoint, higher sensitivity can be attained by increasing the sampling area by introducing multiple internal

reflections of the IRE, or by using thin-film diamond strip MIR waveguide spectroscopy, as we recently have demonstrated [18].

To further analyze the CRP amide I band, procedures for accounting for the binder´s overlapping amide I band must be done. Subtraction of the binder spectrum from the total spectrum, results in the blue spectrum in Fig. 2a, where the amide I and II bands are shown. When comparing this final spectrum with literature data of human CRP [13], it is seen to agree well with a maximum positioned around 1632-1633 $cm^{-1}$. In Fig. 2b the amide I band of this spectrum is compared to the corresponding spectrum obtained from CRP solution dropped directly onto diamond ATR crystal. Evidently, the spectral envelope of the amide I bands are in good agreement. From these findings we conclude that CRP is successfully immobilized on the functionalized NCD surfaces with preserved secondary structure. Previous quantitative analysis of the CRP amide I band has been done with CRP dissolved in $D_2O$ based buffers [13], where it was concluded that $\beta$-sheet structure (1620-1640 $cm^{-1}$) was the dominating secondary structure (50 %), which also is consistent with our results. In contrast, the maximum of amide I of the peptide binder is 1653 $cm^{-1}$, indicating a high content of $\alpha$-helix structure, since its corresponding peak is centered around 1650-1655 $cm^{-1}$. This band agrees well with ATR-FTIR spectrum previously measured on a structural related synthetic peptide possessing 48 amino acids of helix-loop-helix form and an absorbance maximum at 1652 $cm^{-1}$ [8], which shows that the functionalized NCD preserves the protein structure. For CRP, a shoulder on the amide I band is also seen at 1652 $cm^{-1}$ (most pronounced for CRP in buffer, Fig. 2b) in accordance to Dong et al. obtained from spectral deconvolution and second derivatives, assigning the complete amide I band into components of 50 % $\beta$-sheet (1631 and 1695 $cm^{-1}$), 13 % $\alpha$-helix (1654 $cm^{-1}$), 23.3 % turn (1670, 1678 and 1688 $cm^{-1}$) and 13.7 % unordered (1646 $cm^{-1}$). In Fig. 2b the 1690 $cm^{-1}$, due to the dominant $\beta$-sheet, is also distinguishable for CRP on NCD.

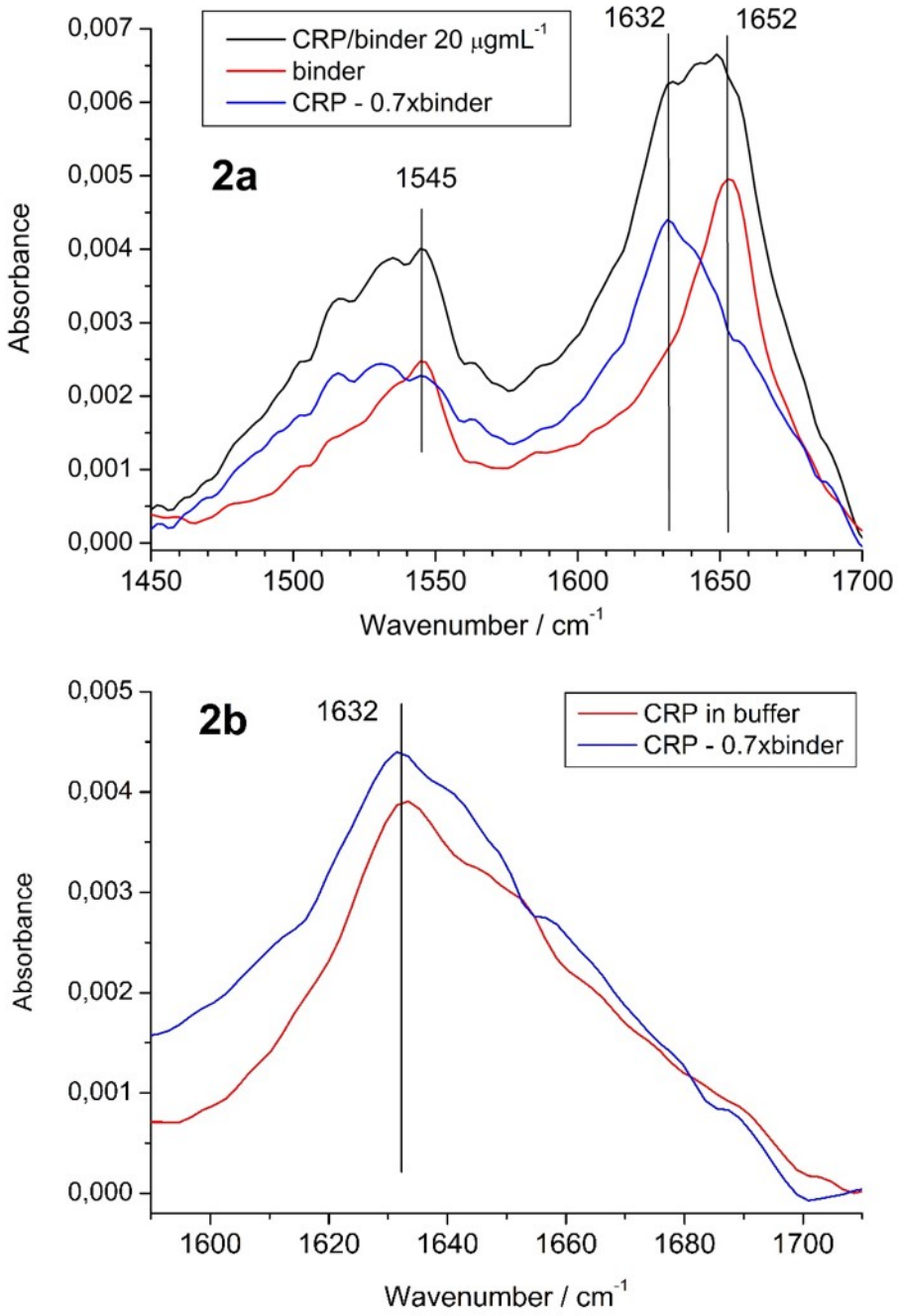


*Fig. 2 Representative ATR-FTIR spectra of amide I and II bands. a) CRP bound to the binder on NCD (black), the binder bound to Pluronic (red) and difference spectrum between CRP (+binder) and binder (blue) multiplied by 0.7. b) Amide I of CRP measured with CRP solution dropped onto ATR-crystal (red), and CRP selectively bound to coated NCD film (blue) calculated by taking the difference between the black (CRP + binder) spectrum and red (binder) spectrum in fig. 2a multiplied by 0.7).*

The pureness of sample solutions was also studies. Buffer solution of CRP was added onto the diamond ATR crystal as liquid droplet (Figs. 2b and Fig. 3). The polypeptide was in powder form and pressed onto the ATR crystal (Fig. 3). In the latter spectrum, peaks between 1100 and 1200 $cm^{-1}$ were observed, which can be attributed to sample impurity. Similar peaks were documented in a related study [8], assigned to trifluoroacetate anion, TFA. This compound is commonly used in purification of

synthetic peptides. Occasionally TFA was observed when synthetic polypeptide was bonded to Pluronic. The subsequent CRP immobilization was in such cases usually incomplete and resulted in poor quality spectra. It is clear that the ATR-FTIR methodology presented here contributes substantially to the understanding of the NCD functionalization steps, and can be used to validate impurity and protein structure.

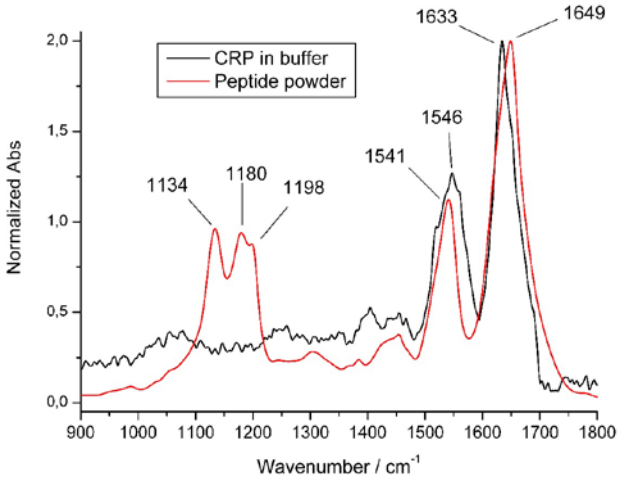


*Fig. 3 ATR-FTIR spectra of CRP aqueous sample (black), and synthetic polypeptide powder containing TFA impurity (red).*

In summary, it is demonstrated that with ATR-FTIR spectroscopy easy and rapid discrimination of sample purity, , and protein structure bound to NCD surfaces can be performed. A previous reported immobilization protocol of CRP adsorption on a synthetic peptide anchored to Pluronic coated NCD was stepwise checked by non-destructively monitoring the ATR-FTIR signature of each layer. The NCD surface, functionalized to selectively capture CRP, was probed by MIR evanescent spectroscopy in a force-controlled anvil-type configuration. The method yields reproducible vibrational spectra, clearly showing the amide bands, which in turn strongly indicate that CRP was bonded to the NCD surfaces without loss of secondary structure. The CRP amide I band showed a dominant β-sheet structure suggesting that the CRP retains its secondary structure upon binding to the functionalized NCD surface. The functionalized NCD surface is thus biocompatible in this respect. The concentration of the CRP in buffer was relatively low, 10-20 μg/mL, compared to other reported IR studies. This was achieved by the hydrophobic character of the Pluronic coated F-terminated NCD surfaces, which effectively remove excess water in the NCD coating, which greatly facilitates amide I bands analyses, even in aqueous protein samples.

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