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Phillip Steindel, Ph.D.

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Dear Phillip Steindel,

We are very grateful for the letter with editorial and reviewers comments. We went through each and every comment and left a detailed response. Some of the comments required significant manuscript amendments and video rerecordings, which were cauciously considered and implemented. We believe new submission fully complies with JOVE's editorial policy and reviewer recommendations.

Hereby we attach the revised manuscript, rebuttal letter, previous manuscript with deletions and additions to track all corrections, four figures and two tables.

Thank you for your time effort.

Yours sincerely,

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TITLE:

Study of Peptide Adsorption on Solution Dispersed Inorganic Nanoparticles Using Depletion Method

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KEYWORDS:

titanium dioxide, anatase, nanoparticles, peptide, adsorption, adsorption model

SUMMARY:

The first step in comprehending biomolecule-inorganic solid phase interaction is revealing fundamental physicochemical constants that may be evaluated by establishing adsorption isotherms. Adsorption from the liquid phase is restricted by kinetics, surface capacity, pH, and competitive adsorption, which all should be cautiously considered before setting an adsorption experiment.

ABSTRACT:

Fundamentals of inorganic-organic interactions are critically important in the discovery and development of novel biointerfaces amenable for utilization in biotechnology and medicine. Recent studies indicate that proteins interact with surfaces through limited adsorption sites. Protein fragments such as amino acids and peptides can be used for interaction modeling between complex biological macromolecules and inorganic surfaces. During the last three decades, many valid and sensitive methods have been developed to measure the physical chemistry fundamentals of those interactions: isothermal titration calorimetry (ITC), surface plasmon resonance (SPR), quartz crystal microbalance (QCM), total internal reflection fluorescence (TIRF), and attenuated total reflectance spectroscopy (ATR).

The simplest and most affordable technique for the measurement of adsorption is the depletion method, where the change in sorbate concentration (depletion) after contact with solution-

dispersed sorbent is calculated and assumed to be adsorbed. Adsorption isotherms based on depletion data provide all basic physicochemical data. However, adsorption from solutions requires longer equilibration times due to kinetic restrictions and sorbents with a high specific surface area, making it almost inapplicable to macroscopic fixed plane surfaces. Moreover, factors such as the instability of sols, nanoparticle aggregates, sorbent crystallinity, nanoparticle size distribution, pH of the solution, and competition for adsorption, should be considered while studying adsorbing peptides. Depletion data isotherm construction provides comprehensive physical chemistry data for literally every soluble sorbate yet remains the most accessible methodology, as it does not require expensive setups. This article describes a basic protocol for the experimental study of peptide adsorption on inorganic oxide and covers all critical points that affect the process.

INTRODUCTION:

For the last 50 years the interaction between inorganic surfaces and peptides has drawn a lot of attention due to its high importance in material science and medicine. Biomedical research is focused on the compatibility and stability of bioinorganic surfaces, which have direct implications for regenerative medicine, tissue engineering[1–3], and implantation[4–7]. Contemporary bioresponsive devices, such as sensors and actuators, are based on functional proteins immobilized on oxide semiconducting surfaces[8–13]. Modern purification practices for protein production often rely on biomolecule interaction properties in downstream purification and separation[14].

Among multiple inorganic oxides, titanium dioxide remains the most utilized in combination with biologically relevant substrates[15,16]. Research in the area of TiO_2-based biointerfaces has concentrated on establishing strong and specific binding of proteins and peptides without changing their biological and structural properties. Ultimately, the major objective is a high surface density layer of biomolecules with high stability and increased functionality that will advance the creation of titanium-based biotechnological and medical applications[17].

Titanium and its alloys have been used extensively as a surgical implant material for at least six decades because a surface TiO_2 layer with a thickness of a few nanometers is corrosion resistant and exhibits a high level of biocompatibility in many in vivo applications[18–20]. Titanium dioxide is also widely considered an inorganic substrate produced in biomineralization, where nucleation and inorganic phase growth accompanied by proteins and peptides may provide materials with promising catalytic and optical properties[21–24].

Given the high relevance of the interaction between inorganic materials and biomolecules in general and protein-TiO_2 interactions in particular, there has been a lot of research to address the manipulation and control of the adsorption of proteins on TiO_2. Due to these studies, some fundamental properties of this interaction have been revealed, such as adsorption kinetics, surface coverage, and biomolecule conformation, giving substantial support for further advances in biointerfaces[5,13].

However, protein complexity adds considerable restrictions on full determination and

understanding of a protein's molecular level interaction with inorganic surfaces. Assuming that the biomolecules interact with the inorganic surfaces through limited sites, some proteins with known structures and amino acid sequences have been reduced to their components— peptides and amino acids— which are studied separately. Some of these peptides have demonstrated significant activity, making them a unique subject of adsorption studies without the need for previous protein separation[25–30].

Quantitative characterization of peptide adsorption on TiO_2 or other inorganic surfaces can be accomplished by means of physical methods that have been adapted specifically for biomolecules for the past few decades. These methods include isothermal titration calorimetry (ITC), surface plasmon resonance (SPR), quartz crystal microbalance (QCM), total internal reflection fluorescence (TIRF), and attenuated total reflectance spectroscopy (ATR), all of which allow for the detection of the adsorption strength by providing key thermodynamic data: The binding constant, Gibbs free energy, enthalpy, and entropy[31].

The adsorption of biomolecules to the inorganic material may be accomplished in two ways: 1) ITC as well as the depletion method use particles dispersed in a solution binding to fixed macroscopic surfaces; 2) SPR, QCM, TIRF, and ATR use macroscopic surfaces modified with inorganic material, such as gold-coated glass or metal chips, quartz crystals, zinc sulfide crystals, and PMMA chips, respectively.

Isothermal titration calorimetry (ITC) is a label-free physical method that measures the heat produced or consumed upon titration of solutions or heterogeneous mixtures. Sensitive calorimetric cells detect heat effects as small as 100 nanojoules, making the measurement of adsorption heat on nanoparticle surfaces possible. Thermal behavior of the sorbate during continuous addition– titration, provides a full thermodynamic profile of the interaction revealing enthalpy, binding constant, and entropy at a given temperature[32–36].

Surface plasmon resonance (SPR) spectroscopy is a surface-sensitive optical technique based on the measurement of the refractive index of the media in close proximity to the studied surface. It is a real-time and label-free method for monitoring reversible adsorption and adsorbed layer thickness. The binding constant can be calculated from the association and dissociation rates. Adsorption experiments performed at different temperatures may provide information about the temperature dependence of the activation energy and sequentially other thermodynamic parameters[37–39].

The quartz crystal microbalance (QCM) method measures the change in the oscillating frequency of piezoelectric crystals during the adsorption and desorption processes. The binding constant may be evaluated from the ratio of the adsorption and desorption rate constants. QCM is used for relative mass measurements and therefore, needs no calibration[25,27,40]. QCM is used for adsorption from both gas and liquid. The liquid technique allows QCM to be used as an analysis tool to describe deposition on variously modified surfaces[41].

Total internal reflection fluorescence (TIRF) is a sensitive optical interfacial technique based on

the measurement of the fluorescence of adsorbed fluorophores excited with internally reflected evanescent waves. The method allows for the detection of fluorescent molecules covering the surface with thicknesses on the order of tens of nanometers, which is why it is used in the study of macromolecular adsorption on various surfaces[42,43]. In situ monitoring of the fluorescence dynamics upon adsorption and desorption provide the adsorption kinetics and hence thermodynamic data[42,43].

Attenuated total reflectance (ATR) was used by Roddick-Lanzilotta to establish lysine adsorption isotherms based on the lysine spectral bands at 1,600 and 1,525 cm^{-1}. This is the first time that the binding constant for a peptide on TiO_2 was determined using an in situ infrared method[44]. This technique was effective in establishing adsorption isotherms for polylysine peptides[45] and acidic amino acids[46].

Unlike the abovementioned methods, where the adsorption parameter is measured in situ, in a conventional experiment the amount of the adsorbed biomolecules is measured by the concentration change after the surface contacted the solution. Because the concentration of a sorbate decays in a vast majority of adsorption cases, this method is referred to as the depletion method. Concentration measurements require a validated analytical assay, which may be based on an intrinsic analytical property of the sorbate or based on the labeling[47–50] or derivatization[51,52] thereof.

Adsorption experiments using QCM, SPR, TIRF, or ATR require special surface preparation of the chips and sensors used for adsorption studies. Prepared surfaces should be used once and require change upon switching the adsorbate, due to the inevitable hydration of the oxide surface or possible chemisorption of a sorbate. Only one sample at a time can be run using ITC, QCM, SPR, TIRF, or ATR, whereas in the depletion method one can run dozens of samples, for which the quantity is only limited by the thermostat capacity and sorbent availability. This is especially important when processing large sample batches or libraries of bioactive molecules. Importantly, the depletion method does not require costly equipment but solely a thermostat.

However, despite its obvious advantages the depletion method requires complex procedural features that may seem cumbersome. This article presents how to perform a comprehensive physicochemical study of dipeptide adsorption on TiO_2 using the depletion method and addresses issues that researchers may face when performing relevant experiments.

PROTOCOL:

1. Preparation of dipeptide stock solutions and dilutions

1.1. Preparation of 16 mM dipeptide solution

1.1.1. Place 0.183 g of a dipeptide (Ile-His) (see **Table of Materials**) in a sterile polymeric test tube, dilute to 35 mL with double-distilled water (DDW), and dissolve at room temperature (RT) under vigorous stirring.

NOTE: If the dipeptide does not dissolve in DDW while stirring, place the dipeptide solution into an ultrasonic bath and sonicate for a few minutes.

1.1.2. Prepare a 50 mM solution of 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer by dissolving 0.533 g of dry 2-(*N*-morpholino)ethanesulfonic acid in 50 mL of DDW in the sterile test tube. Prepare a 50 mM sodium hydroxide solution by dissolving 200 mg of sodium hydroxide in 100 mL of DDW.

1.1.3. Adjust the pH of the predissolved dipeptide solution to 7.4 by carefully adding (microliter titration) 50 mM MES, or 50 mM sodium hydroxide to the 16 mM dipeptide solution, stirring at RT and monitoring the pH with a pH meter. After adjusting the pH, pour the solution into a measuring cylinder, rinse the test tube, and fill the measuring cylinder with DDW to 40 mL to make a final concentration of 16 mM.

1.2. Preparation of dipeptide dilutions from 16 mM stock solution

1.2.1. Prepare peptide dilutions with concentrations between 0.4 and 12.0 mM by diluting the 16 mM dipeptide solution with DDW. For example, in order to prepare an 8 mM dipeptide solution, add 7 mL of DDW to 10 mL of the 16 mM dipeptide solution. After dilution, adjust the pH to 7.4 by adding 50 mM MES or 50 mM NaOH drop by drop to the dipeptide solution (see step 1.1.3). After adjusting the pH, pour the solution into a measuring cylinder, rinse the test tube, and fill the measuring cylinder up to 20 mL with DDW to make the dipeptide concentration 8 mM.

NOTE: Other dilutions of 16 mM dipeptide stock solution with concentrations of 0.4, 0.8, 1.2, 1.6, 2.0, 3.0, 4.0, 8.0, and 12.0 mM, are prepared in accordance with **Figure 1**. The adjustment of each dipeptide solution pH to 7.4 is described in step 1.1.3.

2. Preparation of titania sol

2.1.1. Prepare a 10 mM solution of MES buffer by dissolving 1.066 g of MES in 500 mL of DDW. Adjust the pH to 7.4 with dry sodium hydroxide upon stirring and monitoring the pH with the pH meter.

2.2. Grind 200 mg of nanocrystalline TiO_2 in a mortar for at least 5 min (see **Table of Materials**).

2.3. Weigh 40 mg of the ground titanium dioxide nanoparticles into a laboratory flask. Put the flask into the sonication bath (see **Table of Materials**) using the laboratory stand.

2.4. Add 20 mL of 10 mM MES buffer into the flask with TiO_2 and sonicate in an ultrasonic bath (5 L, 40 kHz, 120 W) for 20 min.

3. Mixing and thermostating

3.1. Set the thermostat (see **Table of Materials**) to the desired temperatures (i.e., 0.00, 10.00, 20.00, 30.00, or 40.00 °C).

3.2. Add 1 mL of the sonicated sol of TiO_2 to the marked adsorption vials. Place the marked adsorption vials against corresponding dipeptide dilution in a makeshift flotation device made of extruded polystyrene foam. Place the flotation device with the marked vials and corresponding dipeptide dilutions into the thermostat for at least 5 min.

3.3. Add 1 mL of each dipeptide dilution to the corresponding marked adsorption vial, making sure all mixing solutions have the same temperature. Keep the series of obtained adsorption samples on the thermostat at 0.00, 10.00, 20.00, 30.00, or 40.00 °C for 24 h to achieve the adsorption equilibrium.

NOTE: Cautiously shake all the samples of obtained dispersions prior to putting them into the thermostat.

3.4. Occasionally mix the TiO_2 dispersions by manually shaking them during thermostating.

4. Filtration of the thermostated samples

4.1. In order to avoid temperature-induced reequilibration take out one sample at a time from the thermostat for filtration.

4.2. Take a sample of the dipeptide solution from each glass vial with a syringe, through a syringe needle. Remove the needle from the syringe and put on the syringe filter (see **Table of Materials**) to filter the dipeptide solution into the glass vial. Repeat the filtration with the other samples.

4.3. Analyze the filtrate in accordance with section 5.

NOTE: Do not centrifuge the samples, because it takes a few minutes and may cause a change in the concentration equilibrium.

5. Derivatization and HPLC analysis

5.1. Make a 50 mL solution of trifluoroacetic acid (TFA) in acetonitrile. Add 0.34 mL of TFA in the measuring cylinder and adjust the volume of the solution to 50 mL with acetonitrile at RT.

CAUTION: Work with TFA under a fume hood with exhaust ventilation, because trifluoroacetic acid is harmful when inhaled, causes severe skin burns, and is toxic for aquatic organisms even at low concentrations[53].

5.2. Prepare the derivatization solution (i.e., Edman reagent[54]) by placing 299 µL of phenyl isothiocyanate and 347 µL of triethylamine in a graduated cylinder and adjusting the volume of

solution to 50 mL with acetonitrile at RT.

5.3. Prior to the high-performance liquid chromatography (HPLC) analysis, derivatize the samples with Edman's reagent in the chromatography vials. Mix 400 µL of the sample with 400 µL of Edman's reagent. Heat the sample at 60 °C for 15 min. After heating, neutralize the sample with 225 µL of the TFA solution and wait for a few minutes to cool the sample to RT.

5.4. Use HPLC analysis (see **Table of Materials**) to determine the concentration of the dipeptide solution before and after adsorption. Place the chromatography vials with the analyzed solutions into the HPLC autosampler and start analyzing the samples with the necessary conditions, which are set by the software (see **Table of Materials**).

NOTE: The mobile phase consists of 0.1% TFA in deionized water and pure acetonitrile, with acetonitrile gradients from 20–90% at 286 nm for 13 min. Analyze each sample in triplicate. Measure the dipeptide solution concentration using the previously established calibration curve (**Figure 2**). For chromatography specifications see Shchelokov et al.[55].

REPRESENTATIVE RESULTS:

Adsorption of a dipeptide on nanocrystalline titanium dioxide was studied at the biocompatible conditions in a temperature range of 0−40 °C. Experimental dipeptide adsorption (A, mmol/g) on the surface of a titanium dioxide was evaluated as

$$A = \frac{V(C_0 - C_e)}{m} \qquad (1)$$

Where C_0 and C_e are the dipeptide starting and equilibrium concentrations in millimoles, respectively; V is the volume of a dipeptide solution in liters; and m is the weight of the sorbent in grams.

The measurements of the dipeptide adsorption were data processed using the Henry model. This isotherm model assumes adsorption at relatively low concentrations with the sorbate molecules isolated from each other on a sorbent surface and is suitable for describing the experimental data (**Figure 3**). Note, however, that this model can only be applied in the case of reversible adsorption, which should be confirmed as well. IR-spectroscopy of the material rinsed multiple times is suitable for this purpose. The obtained equilibrium peptide amounts on the TiO_2 and solution are related in accordance with the linear equation:

$$A = K_H C_e \qquad (2)$$

where K_H is Henry's adsorption constant.

The equilibrium binding constant K_H was obtained from the slope of the dependence of dipeptide adsorption (A) on the dipeptide equilibrium concentration (C_e). The standard Gibbs free energy (ΔG, kJ/mol) for each temperature T was determined through the Van't Hoff

equation:

$$\Delta G = -RT \ln K_H \qquad (3)$$

where *R* is the ideal gas constant in *J*/mol***K*, and *T* is the temperature of the adsorption process in Kelvin.

Dipeptide Gibbs free energies determined at each temperature (**Figure 4**) disclosed enthalpy (ΔH) as an interception of the linear regression with the ΔG axis. The regression variable, the entropy of the process (ΔS), was derived from the fundamental equation:

$$\Delta G = \Delta H - T\Delta S \qquad (4)$$

The calculated values of the equilibrium binding constant (K_H), standard Gibbs energy (ΔG), enthalpy (ΔH), and entropy (ΔS) for Ile-His are presented in **Table 1**.

FIGURE AND TABLE LEGENDS:

Figure 1: Dilution of the 16 mM dipeptide stock solution.

Figure 2: Calibration curve at different dipeptide concentration. The dipeptide concentrations were between 0.4–16.0 mM.

Figure 3: The dipeptide adsorption isotherms calculated by the Henry model for each temperature. Dipeptide adsorption isotherms at (**A**) 0 °C (**B**) 10 °C (**C**) 20 °C (**D**) 30 °C, and (**E**) 40 °C, respectively. The calculated correlation coefficients (R^2) fell into a 0.96−0.99 range for all obtained Henry model isotherms. Error bars represent the 95% confidence interval for each sample concentration measured in triplicate.

Figure 4: Dependence of the standard Gibbs free energy of the dipeptide adsorption on temperature. Error bars represent the 95% confidence interval for Gibbs free energy as indirect measurement based on Henry Model.

Table 1: Thermodynamic parameters of dipeptide adsorption.

DISCUSSION:

Adsorption from solutions for isotherm construction requires a longer time for equilibration due to kinetic restrictions and sorbents with a high specific surface area. Moreover, instability of sols, nanoparticle aggregates, crystallinity, nanoparticle size distribution, pH of the solution, and competition for adsorption should be considered while adsorbing amino acids. However, adsorption isotherm construction using the depletion method remains the most available methodology, because it does not require expensive setups, and yet it provides exhaustive physical chemistry data for literally every soluble sorbate.

A distinction has to be made between adsorption modes (i.e., solution dispersed particles or on

a fixed surface) when a crystalline material is used as a sorbent. One should expect a substantial difference in the distribution of crystalline faces on macroscopically flat surfaces and on particles. Resulting thermodynamic parameters determined from adsorption of peptides on nanoparticles may not correspond to the thermodynamic parameters of peptide adsorption to macroscopically flat surfaces.

The average amount of peptides adsorbed on the inorganic surfaces is extremely low. At room temperature, this value is about several hundreds of micrograms per square meter[28]. This small amount of adsorbate requires accurate measurement methods and solids with well-developed surfaces. Therefore, small particle substances with a large specific surface (hundreds of square meters) should be used for adsorption experiments[43,56–60].

Peptides are, like proteins, unstable, and retain their functionality at a narrow range of conditions. Adsorption experiments were performed on nanocrystalline titanium dioxide at biocompatible temperatures of 0 °C–40 °C (273.15 K–313.15 K), which are similar to those of a normal, functioning, living organism. Adsorption at higher or lower temperatures are irrelevant and should not be considered for the experiment.

Multifunctional biologically active compounds also exhibit high susceptibility to pH of the media, as it affects the surface charge and therefore Coulomb interactions between charged functional groups[61–63]. The sorbent charge of oxide materials is also pH-dependent due to active proton exchange at the hydrated surface[64]. In order to establish pH stable conditions for adsorption equilibrium use of a buffer is required. In this study, MES buffer is used for its noncoordinating property[65], so it would not compete with the peptide for adsorption on the metal oxide surface, unlike phosphate buffers[66].

This recent test of amino acid adsorption shows that the major binding site on the nanoparticle is the surface defect[55]. Defect distribution on the surface is one of the least controllable features of the nanocrystalline substrates, hence one should use sorbent from the same batch in order to maintain consistency in adsorption studies.

QCM, plasmon resonance, and ITC are genuine methods with subtle sensitivity that in a combination of spectroscopic methods reveal structural peculiarities of the adsorbate during interplay with the surface. However, they do not overcome kinetic restrictions and still require considerable time to achieve adsorption equilibration. Furthermore, only one sample at a time can be processed, which makes batch sample analysis challenging. On the other hand, the depletion method presented is simple and only restricted to the thermostat capacity, making the processing of a large number of samples possible.

The thermostated samples should be filtered as soon as they are removed from the thermostat in order to avoid temperature-induced reequilibration. Although equilibration at a new temperature may take up to a few hours, keeping the adsorption samples at a different temperature should be minimized. Centrifugation of the samples for supernatant separation is also not recommended, because it takes up to a few minutes and may cause a change in the

concentration equilibrium. The choice of the filter material depends on the sorbate nature and should reduce possible filter-binding for maximum recovery. It is best to follow vendor instructions and recommendations when choosing specific filters.

Additionally, one should bear in mind that concentration change in the adsorption studies should be monitored using validated quantification method using mass spectrometry, radio-spectroscopy, or UV-visible spectroscopy. The analysis is easy if the adsorbate is spectroscopically active, otherwise additional labeling or derivatization of the adsorbate is required.

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DISCLOSURES:

The authors have nothing to disclose.

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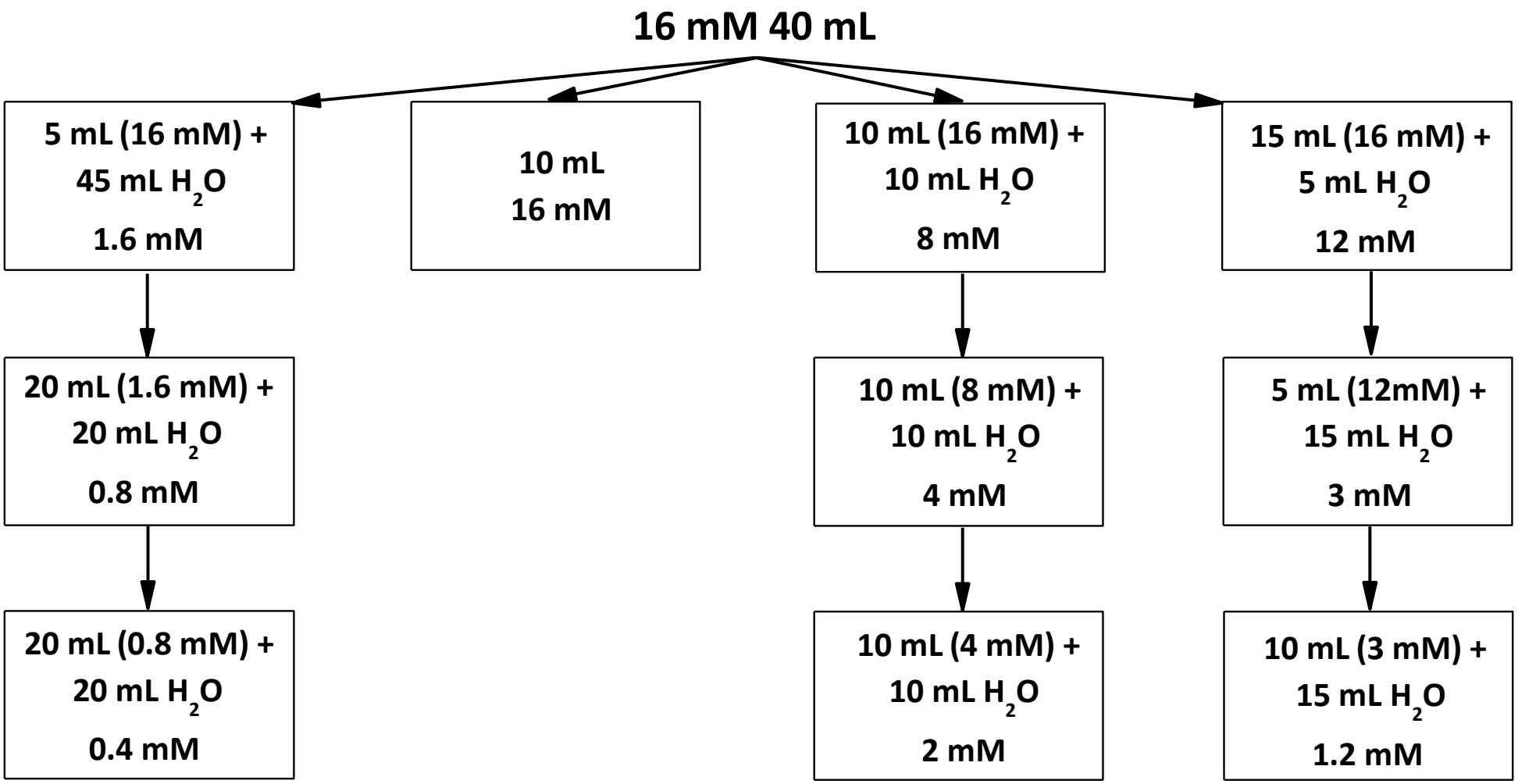
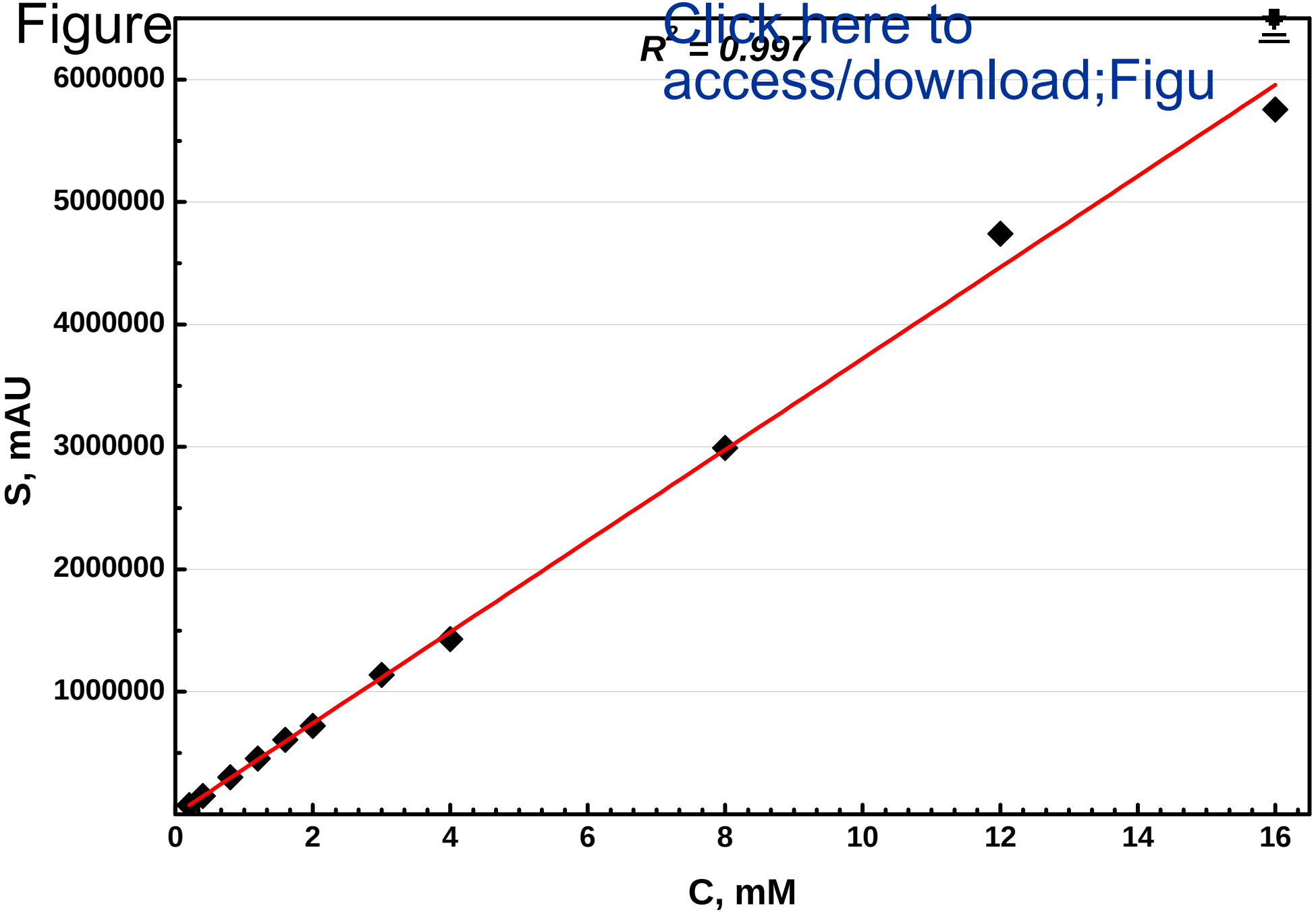
Figure



Figure



Figure

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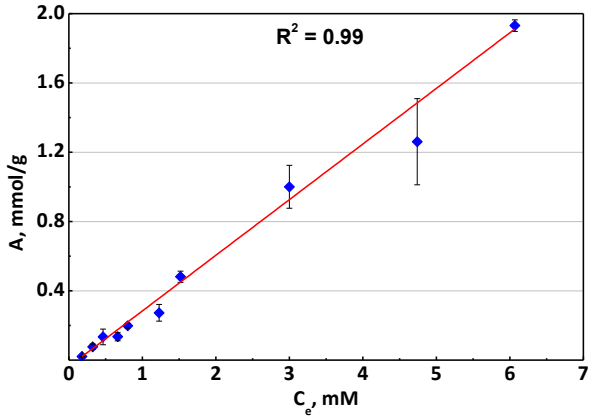
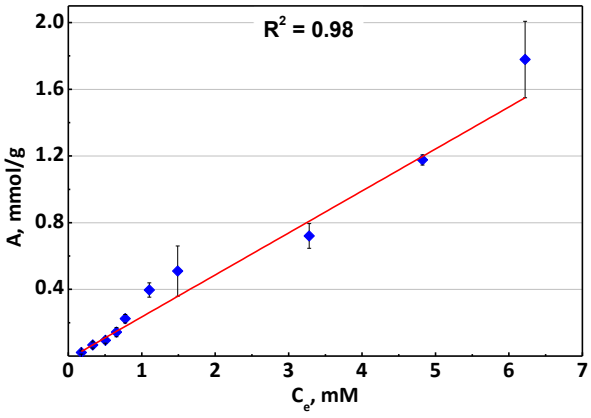


A



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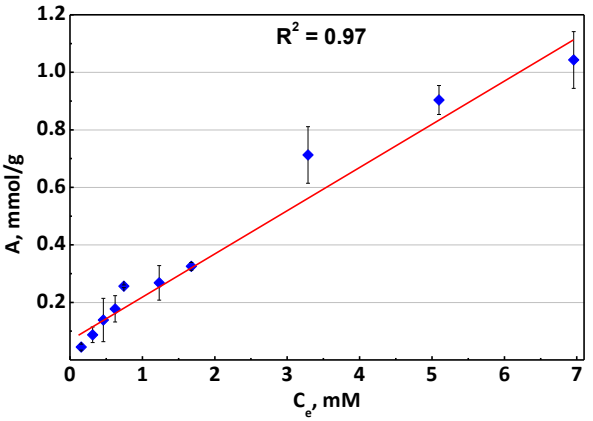

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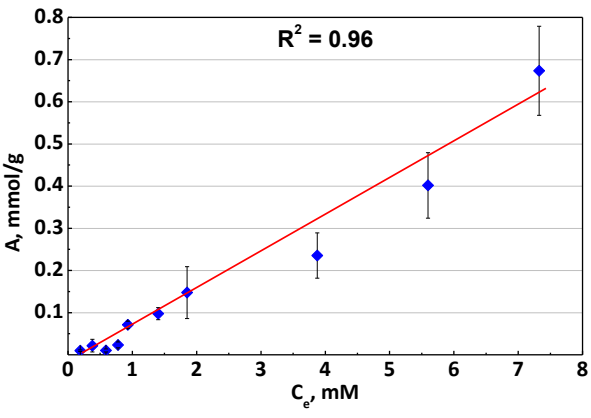

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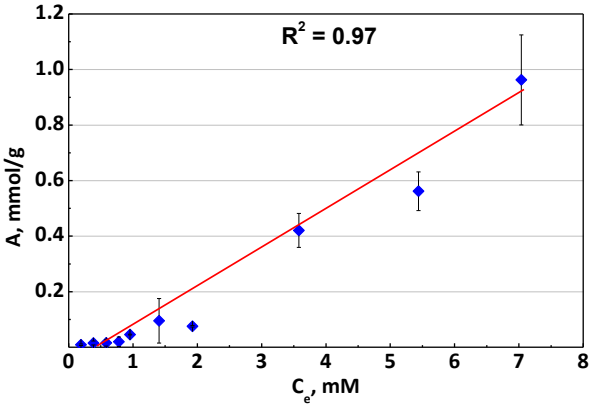

E

Figure

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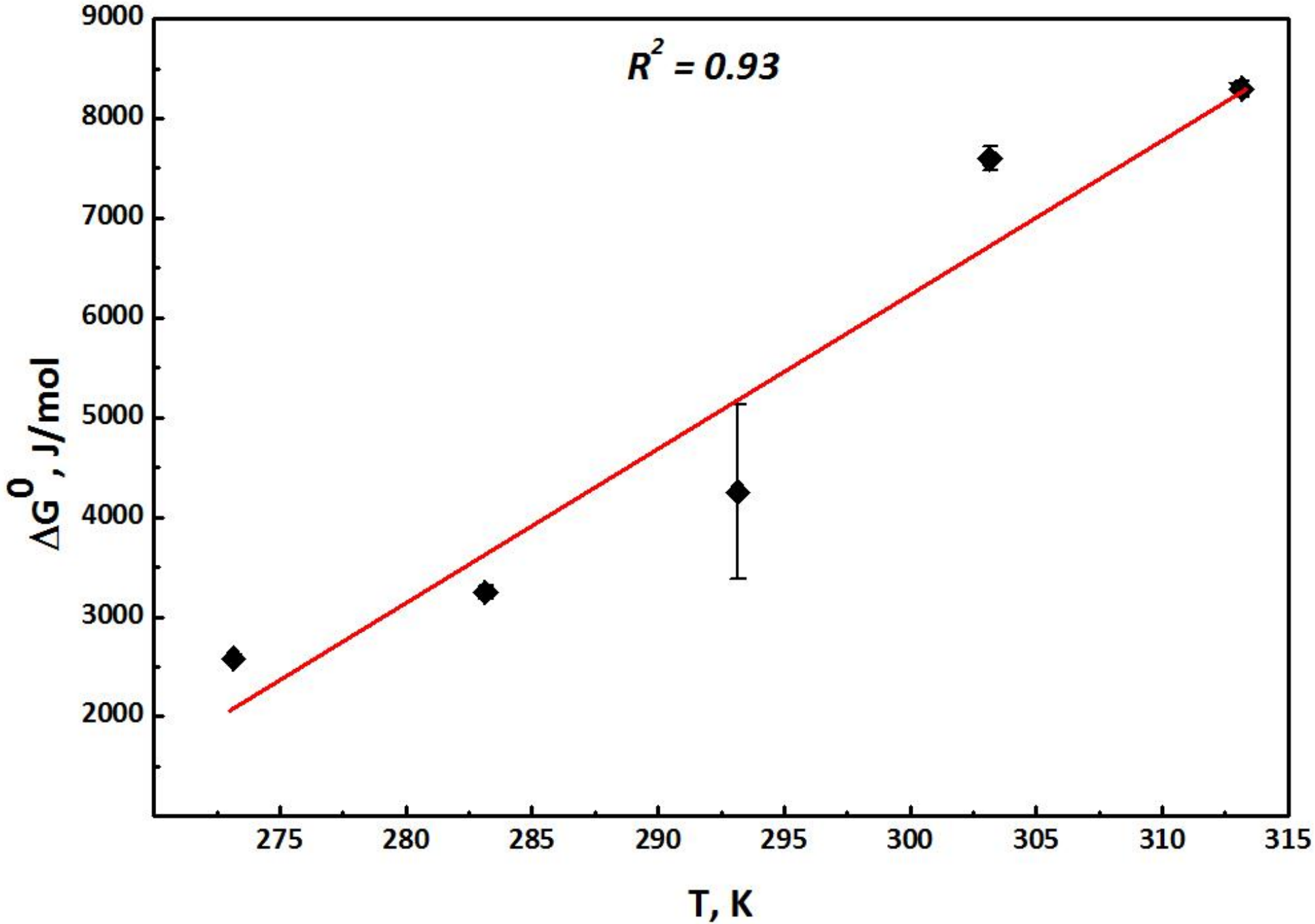

Table 1

T, K	K_H	ΔG^0, kJ/mol	ΔH^0, kJ/mol	ΔS^0, kJ/mol K
273.15	0.32 ± 0.01	2.6 ± 0.0		
283.15	0.25 ± 0.01	3.2 ± 0.1		
293.15	0.17 ± 0.06	4.3 ± 0.9	- 41 ± 9	- 0.16 ± 0.03
303.15	0.050 ± 0.002	7.6 ± 0.1		
313.15	0.037 ± 0.002	8.3 ± 0.1		

Name of Material/ Equipment	Company	Catalog Number
2-(*N* -Morpholino)ethanesulfonic acid	TCI Chemicals	4432-31-9
Acetonitrile	Panreac AppliChem	
Chromatography vials		
Dipeptide Ile-His	Bachem	4000894
Double-distilled water		
		3000865
Heating cleaning bath “Ultrasons-HD”	J.P. Selecta	
High-performance liquid chromatograph system equipped with a UV−vis detector	Shimadzu, LC-20 Prominence	
Isopropanol	Sigma-Aldrich (Merck)	67-63-0
LabSolutions Lite	Shimadzu	223-60410
Nanocrystalline TiO_2		
Phenyl isothiocyanate	Acros Organics	103-72-0
Reversed-phase Zorbax column	ZORBAX LC	
Syringe filter	Vladfilter	
Test sterile polymeric tube		
Thermostat TC-502	Brookfield	
Triethylamine	Sigma-Aldrich (Merck)	121-44-8
Trifluoroacetic acid	Panreac AppliChem	163317

Comments/Description
MES, >98%
HPLC grade
glass
DDW was obtained on spot
5 L, 40 kHz, 120 Watts
HPLC
99.70%
Software for high-performance liquid chromatography system
Pure anatase with at least 99% crystallinity. Average particle size 10.62 ± 3.31 nm. Specific surface 131.9 m2/g (BET). See *Langmuir* 2019, 35, 538−550, for details.
PITC, 98%
150×2.5 mm i.d. with a mean particle size of 5 µm
25 mm, 0.2 µm pore, cellulose acetate polypropylene
Refrigerating/heating circulating bath with the programmable controller for the sample derivatization
TEA; 99%
TFA, 99%

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Sonication settings has been described in brackets

2. 4.2: Please provide examples of appropriate analytical methods (or, do you just mean section 5?).

We referred to the section 5. Corresponding text alteration has been added

Figures:

1. Please remove titles and legends from the Figures themselves.

We have removed all titles and legends from the Figures themselves.

2. Figure 1: Please use 'mL' instead of 'ml'.

Corrected.

3. Figure 3: Please explain the error bars in the legend.

Explanation for the error bars has been provided in the "FIGURE AND TABLE LEGENDS" section.

4. Figure 4: What is the R-squared value here?

Correlation coefficient has been inserted in the plot.

5. Table 1 was not included in the current submission. Please include as an .xls/.xlsx formatted file.

We have included the table in the current submission

References:

1. Please do not abbreviate journal titles.

Previously Abbreviated name of the journal Phys. Chem. Chem. Phys has been rewritten.

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Present table has all information on the materials used for the experiment.

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1. Please ensure the video matches the written protocol as much as possible. In particular, there should not be information in the video that is not in the written material, e.g.:

a. Specific concentrations of peptide are shown (~3:03) in the video, but not given in the protocol.

All specific concentration has been added to the NOTE, below the section1.2.1.

2. ~3:40-3:59- The narration here does not quite line up with the actions shown on screen (e.g. the TiO2 is shown being added well after the narration).

We have adjusted the voice recording to align with the video. (~4:10-4:20)

3. ~6:12-6:28-In the onscreen text, both volumes should be '400 µL' (include a space).

Space has been added between 400 and µL (~7:01-7:18)

4. 0:01, 1:00, 2:05, 3:06, 4:07, 4:45, 5:23, 6:09, 6:47, 7:08-8:13 - The university logo should be removed at the times listed. It can be included at the end of the video.

All University logos has been removed

5. 8:14 - A chapter title card that reads "Conclusion" should be added here.

Chapter card "Conclusions" has been added.

6. It sounds like the sound from the camera has been left in the video. This audio should be removed, as it contains distracting noise.

Noise on the background is caused by permanently working vacuum pumps that cannot be stopped. Since we have significantly rewritten the introduction and the title of the article the corresponding video part was rerecorded accordingly without any distracting noise.

7. 1:00, 2:05, 5:23, 6:09, 6:47 - The chapter title cards often contain both headings (e.g., 1) and subheadings (e.g., 1.2). If the authors wish for the chapters to be visible in our navigable chapter list, we recommend abandoning this naming format and giving each chapter a discrete name. This will ensure that each chapter will appear in the chapter list.

We removed the hierarchical outline numbering in title cards.

8. 1:44, 5:02, 5:52, 5:58, 6:20 - Jump cuts (https://en.wikipedia.org/wiki/Jump_cut), like the one here, where the angle is not changed between shots, should be avoided. Jump cuts tend to have a jarring effect on the audience, and don't fit with our visual style. Please use cross dissolves (aka fades) to cover those edits.

Blur-dissolves (1:44) and zoom-out transitions has been added to soften the Jump-cuts.

1:44 now 1:44 - zoom-out transition

5:02 now 5:52 – rerecorded

5:52 now 6:40 – blur dissolve

5:58 now 6:48 – blur dissolve

6:20 now 7:11 – zoom-out transition

9. 5:15 - We think the narration says "Repeat the with samples..." It sounds like a word is missing. If this is the case, this sentence (the entire sentence) should be rerecorded.

Now 6:03. Narration has been rerecorded: “Repeat the filtration with samples of other concentrations…”.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Depletion studies for determining Gibb's free energy are presented. However, this technique is plagued with problems.

Major Concerns:

1. time: 1:10, 2:04, 2:46 - graduated cylinder should be used for this dilution, especially for specific volumes. The markings on the centrifuge tubes are not accurate enough.

Use of the centrifuge tubes is convenient when adjusting pH of the solution is required. It is often hard to fit pH-electrode into measuring cylinder. Once the pH is adjusted in the centrifuge tube, subsequent refill up to specific volume using cylinder may cause inevitable losses and significant error in final concentration.

We have rerecorded this part adding the volume adjustment using measuring cylinder with additional rinsing of the centrifuge tube. Now 2:00 and 2:55.

2. time 4:22 - temperature setup should be set prior to mixing; all solutions should be thermostated prior to mixing due to the fact that peptide adsorption is so quick

In the case of the irreversible adsorption or chemisorption preheating samples to the specific temperature for adsorption studies is justified. In our case adsorption is reversible which is confirmed with adsorption energy (less than 70 kJ/mol) and IR spectroscopy of the sorbent. (Separate studies now being conducted).

Achieving adsorption equilibrium on nanocrystalline material is never quick. Even for gases, specifically for nitrogen, achieving of adsorption equilibrium takes minutes or dozens of minutes. Considering the adsorption is reversible and takes long time mixing samples few moments prior to 24 h thermostating does not affect resulting equilibrium concentration.

However, considering the uncertainty of the adsorption nature between sorbent and sorbate the samples surely have to be thermostated to the particular temperature to reduce the possible temperature induced adsorption effects. We have rerecorded this part of the video (4:27) and rewritten section 3 of the PROTOCOL accordingly.

3. time 5:03 - SAFETY ALERT - never ever recap a needle due to sticking hazards (it is an outlawed practice)

This is surely the violation of safety rules, so we have rerecorded this part of the video. (5:50)

4. time 5:09 - Filtering of this material is a problem; how do you control the free peptide in solution from adsorbing to the filter itself? Since you are doing a depletion experiment, this is a massive issue with this protocol.

Syringe filters were chosen in accordance with the filter material properties: cellulose acetate exhibits least known peptide or protein binding properties among all filter materials. Following sentences have been added to the DISCUSSION section: "*Choice of the filter material depend on the sorbate nature and should reduce possible filter-binding for the maximum recovery. We recommend following the vendor instructions on the choice of the specific filters."*

Also, since this work is a part of the bigger studies being conducted by graduate students, we have studied syringe filters on their affinity toward 12 dipeptides at the low concentrations (0.2 mM). None of the studied peptide solution exhibited any significant drop in the concentration after filtration with the filters used for this article.

5. It would be common to consider a rinse step of some sort to remove loosely bound peptides from the substrates as well.

Peptide concentration equilibrium achieved between the surface and the volume after prolonged thermostating is enough to establish the isotherm plot. Loosely bound peptides is not an accurate term. Are these peptides extracted from pore volume or desorbed from the surface? If peptides are from pore volume, then rinsing is an unnecessary step as concertation in pores is assumed to be equal to the concentration in the volume. If they are rinsed from surface, then it is not an equilibrium concentration being analyzed.

6. time 8:00 - error analysis extension to DeltaG; are these significant in their differences or do the errors propagate? to not have errors in the DeltaG is a major problem.

Below is included the confidence intervals calculation procedure for the Gibb's free energy, enthalpy and entropy. Calculated confidence intervals are included into Table 1. We did not include this procedure in the manuscript as it is only applicable for Henry Model case and is irrelevant to the manuscript major goal.

Calculation of the Confidence Interval for the Adsorption Gibbs Free Energy

The measurements of dipeptide adsorption were data processed using the Henry model. The obtained equilibrium peptide amounts on the TiO_2 and solution are related in accordance with the linear equation:

$$A = K_H C_e$$

The equilibrium constant K_H is the slope $a = tg\alpha$ from linear equation $y = ax + b$, $b = 0$.

Relative standard deviation S_r of the calculated equilibrium constant *K* was found according to the propagation of uncertainty law:

$$S_r = \sqrt{\left({S_a}/{a}\right)^2 + \left({S_b}/{b}\right)^2}$$

Variances S_a and S_b for the linear regression coefficients *a* and *b,* respectively were found as:

$$S_a^2 = \frac{S_{xy}^2 * \sum x_i^2}{\mathrm{n}\sum x_i^2 - (\sum x_i)^2}$$

$$S_b^2 = \frac{n * S_{xy}^2}{\mathrm{n}\sum x_i^2 - (\sum x_i)^2}$$

, where S_{xy}^2 is the distribution variance:

$$S_{xy}^2 = \frac{\sum (y_i - Y_i)^2}{\mathrm{n} - 2}$$

, where y_i and Y_i, are actual and calculated from regression values, respectively.

The standard deviation of the equilibrium constant S_K was subsequently derived as:

$$S_K = S_r * K$$

The confidence interval for the equilibrium constant *K* at the 95% confidence level was calculated as:

$$CI_K = \frac{S_K * t}{\sqrt{n}}$$

where t is a critical value of Student's t distribution with n-2 degree of freedom.

Using the Van't Hoff equation:

$$\Delta G = -RT * \ln(K)$$

Uncertainty of the Gibbs free energy as the indirect measurement:

$$d\Delta G = -RT\frac{dK}{K}$$

, where *R* is the ideal gas constant, J/mol*K; *T* is the temperature of the adsorption process, K.

The confidence interval for the Gibbs free energy:

$$CI_{\Delta G} = -RT * \frac{(CI_K)}{K}$$

Calculation of the Confidence Interval for the Adsorption Enthalpy and Entropy.

We used the fundamental equation (5), considering enthalpy and entropy of the adsorption are constant at the studied temperatures.

$$\Delta G = \Delta H - T\Delta S \qquad (5)$$

Building the graph in the $\Delta G/T$ coordinates, we established the linear regression of $y = ax + b$ type, where *a* and *b* corresponded to *ΔS* and *ΔH,* respectively, while the variable x corresponded to *T.* from which we derived the distribution variance:

$$S_{xy}^2 = \frac{\sum(y_i - Y_i)^2}{\mathrm{n} - 2}$$

, where y_i and Y_i, are actual and calculated from regression values, respectively

Variances S_a and S_b for the linear regression coefficients *a* and *b* were found as:

$$S_a^2 = \frac{S_{xy}^2 * \sum x_i^2}{\mathrm{n}\sum x_i^2 - (\sum x_i)^2}$$

$$S_b^2 = \frac{n * S_{xy}^2}{\mathrm{n}\sum x_i^2 - (\sum x_i)^2}$$

Confidence intervals for the coefficients *a* and *b*, or entropy and enthalpy respectively, at the 95% confidence level were calculated as:

$$CI_a = \ CI_{\Delta H} = \frac{S_a * t}{\sqrt{n}}$$

$$CI_b = \ CI_{\Delta S} = \frac{S_b * t}{\sqrt{n}}$$

where t is a critical value of Student's t distribution with n-2 degree of freedom.

Reviewer #2:

Manuscript Summary:

The issue is interesting. The information is complete and the experiments can be repeated by other authors

Minor Concerns:

the author suggested some quantification method to validate the results, they could mention which of them they use and show the results

In this manuscript we referred to the quantification method in relation with the necessity to measure the change in dipeptide concentration. No additional method is required for the results validation. Results themselves are separate and independent data, which may be used as additional information for the description of biomolecule-inorganic surface interactions.

Reviewer #3:

Manuscript Summary:

The authors present methods detailing experimental procedures for the adsorption of short peptides to inorganic nanoparticles that are suspended in solution. The methods presented are very good, but the authors need to emphasize that the methods presented only apply to nanoparticles in solution and not to fixed macroscopically sized inorganic surfaces. There are several specific comments listed below that the authors need to address before this manuscript should be considered suitable for publication.

Major Concerns:

1. Page 2, Title. The authors paper only addresses peptide adsorption on inorganic nanoparticles suspended in solution, but not to fixed inorganic surfaces. The title needs to be amended to make this distinction.

The title of the article has been changed: "Study of Peptide Adsorption on Solution Dispersed Inorganic Nanoparticles Using Depletion Method".

2. Page 2 (Title Page), Abstract, lines 42-43 and page 3, lines 48-49. Here the authors state that spectroscopic and computer-based approaches have been the dominant methods for studying peptide adsorption and that these methods only provide qualitative and not quantitative evaluation. This is incorrect. Three of the most commonly used methods for studying peptide adsorption over the past couple of decades have been via isothermal titration calorimetry (ITC), surface plasmon resonance (SPR) spectroscopy, and quartz crystal microbalance (QCM).

Agreed, depletion method being the common physical chemistry quantitative method is irrelevant statement. We have rewritten this part of the abstract. Note, however, that for short peptides computer simulation and spectroscopic methods remain to be dominant in describing interactions peptide and inorganic nanoparticle. We refer to the review on the topic [1], where the ITC and SPR were not mentioned at all and SPR was only mentioned twice.

1. Costa, D., Savio, L. & Pradier, C. M. Adsorption of Amino Acids and Peptides on Metal and Oxide Surfaces in Water Environment: A Synthetic and Prospective Review. *Journal of Physical Chemistry B* **120**,)29(7039–7052 (2016).

Each of these methods can provide quantitative measurement of peptide adsorption isotherms—in fact the authors go into a discussion of these other methods near the end of the introduction.

Authors provide brief description of each method on how basic physical-chemical constants may be extracted followed by discussion making together the half of the introduction

When contrasting the depletion method to methods like ITC, SPR, and QCM later on in the paper, the authors make the point that the difference between depletion and ITC, SPR, and QCM is primarily that that ITC, SPR, and QCM require that samples must be tested one at a time. However, they do not state that these methods are only 'qualitative' as implied in the introduction.

Qualitative character of the spectroscopic (ATR-FTIR, XPS, RAMAN, NMR) and computer based methods (DFT, MD), dominant in characterization of the short peptide-inorganic material interaction was mentioned in the abstract, and did refer to QCM, SPR, TIRF or ATR.

The authors then go on in the introduction (lines 48-49) to state that the most common technique for measurement of adsorption is the depletion method, which is again not correct.

We agree and already stated that ITC, QCM, SPR currently are the most common quantitative physical chemistry methods.

From these statements, it is apparent that the authors are referring only to peptide adsorption on particles dispersed in solution (as used for depletion studies) as opposed to fixed surfaces (as used for SPR and QCM studies).The authors need to clearly indicate that they are addressing peptide adsorption to nanoparticles in solution, not peptide adsorption to fixed surfaces, and they need to properly state the differences instead

As for the fixed surfaces and solution dispersed particles, we agree that the distinction is required for clarity. Additional paragraph has been added to the abstract covering this important adsorption mode distinction:

“Practically, adsorption of biomolecules towards inorganic material may be accomplished in two modes: via solution dispersed particles and towards fixed macroscopic surfaces. ITC, as well as depletion method, often applied in the case of solution dispersed particles, while SPR, QCM, TIRF, and ATR use macroscopic surfaces modified with inorganic material: gold-coated glass or metal chips, quartz crystals, zinc sulfide crystals, and PMMA chips, respectively.”

instead of saying methods like ITC, SPR, and QCM provide only qualitative data.

Nowhere in the submitted manuscript authors have stated that ITC, SPR, and QCM provide only qualitative data.

3. Page 3, abstract, lines 56-58. The authors need to clarify that they are only addressing peptide adsorption to nanoparticles suspended in solution, but not to fixed surfaces.

Lines 56-60 has been amended in order to clarify that depletion method in only applicable towards solution dispersed sorbents.

4. Page 4, line 80. It is incorrect and misleading to state that titanium oxide exhibits bioinert behavior in vivo. TiO2 strongly adsorbs proteins and elicits a subsequent biological response—thus it is NOT 'bio'inert. This statement should be changed to something like 'exhibits a high level of biocompatibility in many in vivo applications' or 'exhibits a high degree of chemical stability'.

Scientific literature has multiple examples of referring to titanium dioxide as to “bioinert” material, and we tried to stick to that medically generalized term. However, the reviewer is right, so we have added the phrase “'exhibits a high level of biocompatibility in many in vivo applications” into the text.

5. Page 10, lines 303-305. The authors need to add to these conditions that the adsorption process must be completely reversible, which is thus limited to short peptides. They should include that the reversibility of the adsorption process should be confirmed prior to the application of the presented analysis method. If the adsorption process is not completely reversible, then thermodynamic parameters cannot be reliably calculated from the apparent adsorption isotherm.

We have rewritten the protocol (lines 230-247) in order to resolve the uncertainty of adsorption nature. Also, the reversibility of the peptide adsorption can be confirmed with IR-spectroscopy after multiple rinsing the peptide-adsorbed TiO2, or any other inorganic material. Once the reversibility of the adsorption has been confirmed, the best-fit adsorption model can be applied. In our case adsorption is reversible which is confirmed with adsorption energy (less than 70 kJ/mol) and IR spectroscopy of the sorbent. (Separate studies now being conducted). Corresponding part in the representative results has been added. (lines 332-334)

6. Page 11, Discussion Section. The authors need to add the issue that the distribution of crystalline faces in TiO2 nanoparticles can be expected to substantially differ from the distribution of crystalline faces on macroscopically flat surfaces of TiO2. Thus, the thermodynamic parameters determined from adsorption studies of peptides on nanoparticle TiO2 surfaces may not be representative of the thermodynamic parameters mediating peptide adsorption to macroscopically flat TiO2 surfaces.

Additional paragraph has been added to address the issue in the discussion Section:

“Important distinction has to be made between adsorption modes: on solution dispersed particles and on fixed surface, when nanocrystalline crystalline material is used as a sorbent. One should expect substantial difference in distribution of crystalline faces on macroscopically flat surfaces and on particles. Resulting thermodynamic parameters determined from adsorption of peptides on nanoparticles may not correspond to the thermodynamic parameters of peptide adsorption to macroscopically flat surfaces.”

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TITLE:

Study of Peptide Adsorption on Solution Dispersed Inorganic ~~Surfaces~~ Nanoparticles Using Depletion Method.

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KEYWORDS:
Titanium dioxide, anatase, nanoparticles, peptide, adsorption, adsorption model

SUMMARY:

The first step in comprehending biomolecule – inorganic ~~surface~~ solid phase interaction is revealing fundamental physicochemical constants which may be evaluated through establishing adsorption isotherms. Adsorption from liquid phase is restricted with kinetics, surface capacity, pH and competitive adsorption, which all should be cautiously considered before setting the adsorption experiment.

ABSTRACT:

Fundamentals of the inorganic-organic interactions are critically important in the discovery and development of the novel biointerfaces amenable for utilization in biotechnology and medicine. Recent studies indicate that proteins interact with surfaces through limited adsorption sites. Amino acids and peptides as molecular bits of proteins can be used for interaction modeling between sophisticated biological macromolecules and inorganic surfaces. During the last three decades, many genuine and sensitive methods have been developed to measure ~~a range of properties that quantify these types of interactions.multiple attempts have been made in order~~

~~to reveal adsorption features of peptide adsorption. However, the dominance of the spectroscopic and computer-based approaches left most of the findings described qualitatively, without quantitative evaluation~~. ~~P~~physical chemistry fundamentals of those intearctions: Isothermal titration calorimetry (ITC), surface plasmon resonance (SPR), quartz crystal microbalance (QCM), total internal reflection fluorescence (TIRF) and attenuated total reflectance spectroscopy (ATR). ~~Adsorption constant, Gibbs free energy, enthalpy, entropy and limiting adsorption remain basic biomolecule inorganic affinity descriptors, which could be revealed from abovementioned methods.~~

The simplest and affordable ~~most common~~ technique for the measurement of the adsorption is the depletion method, where the change in sorbate concentration (depletion) after contact with solution dispersed sorbent is calculated and assumed to be adsorbed. Adsorption isotherms based on depletion data provide all basic physico-chemical data. However, adsorption from solutions ~~for isotherm construction~~ requires longer times for equilibration due to kinetic restrictions and sorbents with the high specific surface area, making it almost inapplicable to macroscopic fixed plane surfaces. Moreover, instability of soles, nanoparticle aggregates, sorbent crystallinity, nanoparticle size distribution, pH of the solution, competing for adsorption, etc. should be considered while adsorbing peptides. And yet, depletion data ~~adsorption~~ isotherm construction remains to be the most available methodology that does not require expensive set-ups, providing exhaustive physical chemistry data for literally every soluble sorbate. In this article, we describe a basic protocol for the experimental study of the peptide adsorption on inorganic oxide, covering all critical points that affect the process.

INTRODUCTION:

For the last 50 years interaction of inorganic surfaces and peptides dragged much of attention due to the high importance of such phenomenon in various fields of material science and medicine. Biomedical research is focused on compatibility and stability of bio-inorganic surfaces, which have direct implications for regenerative medicine, tissue engineering[1–3] and implantation[4–7]. Contemporary bioresponsive devices, such as sensors and actuators, are based on proteins immobilized on oxide semiconducting surfaces without loss of functionality[8–13] Modern purification practices for protein production often rely on the biomolecule interaction properties in downstream purification and separation[14].

Among multiple inorganic oxides, titanium dioxide remains as the most utilized in combination with biologically relevant substrates[15,16]. Research in the area of TiO_2 based bio-interfaces has been concentrated on establishing strong and specific binding of proteins and peptides with a limited impact on biological and structural properties thereof. Ultimately, the major objective in this area is a high surface density layer of biomolecules with high stability and increased functionality that will further advance the elaboration of titanium-based biotechnological and medical applications[17].

Titanium and its alloys have been widely expoited as surgical implant material for at least six decades because a surface TiO_2 layer with a thickness of a few nanometers demonstrates the material's corrosion resistance and exhibits a high level of biocompatibility in many in vivo applications ~~exhibits bioinert behavior in vivo~~ [18–20]. Titanium dioxide is also widely considered as inorganic substrate produced in biomineralization, where nucleation and inorganic phase growth accompanied by proteins and peptides may provide materials with promising catalytic and optical properties[21–24].

Given the high relevance of inorganic–biomolecule interaction in general and protein-TiO_2 interactions in particular major research efforts were addressed to manipulation and control over the adsorption of proteins on TiO_2. During these studies some fundamental properties have been revealed, such as adsorption kinetics, surface coverage, biomolecule conformation, etc., giving substantial support for further advances in biointerfaces[5,13].

However, protein complexity applies considerable restrictions on full determination and understanding their molecular-level interaction with inorganic surfaces. Assuming the biomolecules interact with the inorganics through limited sites some of the proteins with known structure and amino acid sequence have been reduced to its lower bits – peptides and amino acids, which were studied separately. Some of these protein molecular bits – peptides, have demonstrated a significant activity themselves, making them a unique object of adsorption studies without the need for the original protein separation[25–30].

Quantitative characterization of the peptide adsorption on TiO_2 or other inorganic surfaces can be accomplished by means of physical methods, that have been adapted specifically for biomolecules for over past few decades. These methods include Isothermal titration calorimetry

(ITC), surface plasmon resonance (SPR), quartz crystal microbalance (QCM), total internal reflection fluorescence (TIRF) and attenuated total reflectance spectroscopy (ATR), which all allow for adsorption strength revelation by providing key thermodynamic data: binding constant, Gibbs free energy, enthalpy and entropy [31].

Practically, adsorbtion of biomolecules towards inorganic material may be accomplished in two modes: via solution dispersed particles and towards fixed macroscopic surfaces. ITC as well as depletion method oftenly applied in case of solution dispersed particles, while SPR, QCM, TIRF, and ATR use macroscopic surfaces modified with inorganic material: gold coated glass or metal chips, quarz crystals, zinc sulfide crystals, and PMMA chips, respectively.

Isothermal titration calorimetry is a label-free physical method that measures the heat which is produced or consumed upon titration of solutions or heterogeneous mixtures. Sensitive calorimetric cells detect heat effects as small as 100 n*J* making possible measurement of adsorption heat on nanoparticle surfaces. Thermal behavior of the sorbate during continuous addition – titration, provides a full thermodynamic profile of the interaction elucidating enthalpy, binding constant, and entropy at a given temperature[32–36].

Surface plasmon resonance (**SPR**) spectroscopy is a surface-sensitive optical technique based on the measurement of the refractive index of the media in close proximity to the studied surface. It is a real-time and label-free method for monitoring the reversible adsorption, and adsorbed layer thickness. The binding constant can be calculated from association and dissociation rates. Adsorption experiments performed at different temperatures may provide temperature dependence of activation energy and sequentially other thermodynamic parameters[37–39].

The quartz crystal microbalance (QCM) method measures the change in the oscillating frequency of piezoelectric crystals during adsorption and desorption processes. Binding constant may be evaluated from the ratio of the adsorption and desorption rate constants. The QCM is used for relative mass measurements and therefore, needs no calibration[25,27,40]. QCM technique is developed for adsorption from both gas and liquid. The fluid operation gives the opportunity to utilize QCM as an analysis tool to deposition on variously modified surfaces[41].

Total internal reflection fluorescence (**TIRF**) is a sensitive optical interfacial technique based on the measurement of the fluorescence of adsorbed fluorophores that are excited with internally reflected evanescent waves. The method allows for detection of fluorescent molecules covering the surface with tens of nm thickness, which is why it was mostly used in the study the macromolecular adsorption on various surfaces[42,43]. In situ monitoring of the fluorescence dynamics upon adsorption and desorption provide with adsorption kinetics and hence thermodynamic data[42,43].

Use of **attenuated total reflectance** (**ATR**) has been used by a group of Roddick-Lanzilotta in order to establish lysine adsorption isotherms based on the lysine spectral bands at 1600 and 1525 cm^{-1}. This is the first time that the binding constant for peptide on TiO_2 has been determined

using an *in situ* infrared method[44]. This technique was proven effective in establishing adsorption isotherms for polylysine peptides[45] and acidic amino acids[46].

Unlike in abovementioned methods, where adsorption parameter is measured in situ, in a conventional experiment, the amount of the adsorbed biomolecules is measured by the concentration change after the surface contacted the solution. Since the concentration of a sorbate decay in a vast majority of adsorption cases this method is referred to as the depletion method. Concentration measurement requires validated analytical assay, which may be based on an intrinsic analytical property of the sorbate or based on the labeling[47–50] or derivatization[51,52] thereof.

Adsorption experiments using QCM, SPR, TIRF or ATR require special surface preparation of the chips and sensors for adsorption studies. Prepared surfaces should be used once and require change upon switching the adsorbate, due to inevitable hydration of the oxide surface or possible chemisorption of a sorbate. Only one sample at a time can be run using ITC, QCM, SPR, TIRF or ATR, whereas in depletion method one can run dozens of samples, which quantity is only limited with thermostat capacity and sorbent availability. This is especially important during processing large sample batches or libraries of bioactive molecules. And last but not least, the depletion method does not require costly equipment, except the thermostat.

However, despite its obvious advantages depletion method implies complex of procedural features which one may suggest as a considerable obstacle. In this article, we present a comprehensive physicochemical study of dipeptide adsorption on TiO_2 revealing all hidden reefs which researchers may face while performing relevant experiments.

PROTOCOL:

1 Preparation of Dipeptide Stock Solutions and Dilutions

1.1. Preparation of 16 mM dipeptide solution

1.1.1. Place 0.183 g of a dipeptide (Ile-His) (see **Table of Materials**) in the sterile polymeric test-tube and dilute up to 3~~8~~5 mL with double-distilled water (DDW) and dissolve at the room temperature under vigorous stirring.

NOTE: If dipeptide doesn't dissolve in DDW under stirring, place the dipeptide solution into the ultrasonic bath and sonicate for a few minutes.

1.1.2. Prepare 50 mM solution of MES-buffer by dissolving 0.533 g of dry 2-(*N*-morpholino)ethanesulfonic acid in 50 mL DDW in the sterile test tube. Accordingly, prepare 50 mM sodium hydroxide solution by dissolving 200 mg of sodium hydroxide in a 100 mL of DDW.

1.1.3. Adjust pH of the predissolved solution of a dipeptide to 7.4 by cautious adding (microliter titration) solution of MES (50 mM), or NaOH (50 mM) to the dipeptide solution (16 mM) upon stirring at room temperature and monitoring the pH with pH-meter. After adjusting the pH ~~fill the test-tube~~ pour the solution into measuring cylinder, rinse the test-tube and fill the measuring cylinder with DDW up to 40 mL, to make a final concentration of 16 mM.

1.2. Preparation of dipeptide dilutions from 16 mM stock solution

1.2.1. Prepare peptide dilutions with concentrations between 0.4 and 12.0 mM by diluting of 16 mM dipeptide solution with DDW. For example, in order to prepare 8 mM dipeptide solution, add ~~9~~ 7 mL DDW to 10 mL 16 mM dipeptide solution. After dilution, adjust pH to 7.4 by adding dropwise a solution of MES (50 mM) or NaOH (50 mM) to the dipeptide solution (see 1.1.3). After adjusting the pH pour the solution into measuring cylinder, rinse the test-tube and fill the measuring cylinder ~~fill the volume of the solution~~ up to 20 mL with DDW, to make dipeptide concentration of 8 mM.

NOTE: Other dilutions of 16 mM dipeptide stock solution with concentrations of 0.4, 0.8, 1.2, 1.6, 2.0, 3.0, 4.0, 8.0, and 12.0 mM, are prepared in accordance with **Figure 1**. The adjustment of each dipeptide solution pH to 7.4 is described in 1.1.3.

2 Preparation of Titania Sole

2.1. Preparation of 10 mM solution of MES-buffer

Dissolve 1.066 g dry 2-(*N*-morpholino)ethanesulfonic acid by in 500 mL of DDW. Adjust the pH to 7.4 with dry sodium hydroxide upon stirring and monitoring the pH with the pH meter.

2.2. Grind 200 mg of nanocrystalline TiO_2 in a mortar for at least 5 minutes (see **Table of Materials**).

2.3. Weighing of TiO_2

Weigh 40 mg of the ground titanium dioxide nanoparticles into the laboratory flask. Put the flask into the sonication bath (see **Table of Materials**) using the laboratory stand.

2.4. Sonication

Add 20 mL of 10 mM MES-buffer solution into the flask with TiO_2 and sonicate in an ultrasonic bath(5 L, 40 kHz, 120 Watts) for 20 min.

3 Mixing and Thermostating

3.1. ~~Mixing of the sole and dipeptide dilution~~ Setting up of the thermostat

~~Add 1 mL of the sonicated sole of TiO_2 to the 1 mL of each dipeptide dilution concentration.~~ Set the thermostat (see Table of Materials) to the desired temperature 0.00, 10.00, 20.00, 30.00 or 40.00 °C.

3.2. ~~Setting up of the thermostat~~ Thermostating the sole and dipeptide dilutions.

~~Set the thermostat (see **Table of Materials**) to the desired temperature 0.00, 10.00, 20.00, 30.00 or 40.00 °C.~~ Add 1 mL of the sonicated sole of TiO2 to the marked adsorption vials. Place the marked adsorption vials against corresponding dipeptide dilution in an improvised flotation device made of Styrofoam. Place the flotation device with the marked vials and corresponding dipeptide dilutions into the thermostat for at least 5 minutes.

3.3. ~~The adsorption~~ Mixing and thermostating

~~Place the series of adsorption samples to the thermostat at 0.00, 10.00, 20.00, 30.00 or 40.00 °C for 24 h to achieve the adsorption equilibrium.~~ Add 1 mL of each dipeptide dilution to the corresponding marked adsorption vial, making sure all mixing solutions have same temperature. Keep the series of obtained adsorption samples to the thermostat at 0.00, 10.00, 20.00, 30.00 or 40.00 °C for 24 h to achieve the adsorption equilibrium.

NOTE: Cautiously shake all the samples of obtained dispersions prior to putting into the thermostat.

3.4. Agitating

Occasionally agitate TiO_2 dispersions during thermostating.

4 Filtration of the Thermostated Samples

4.1 In order to avoid temperature-induced reequilibration take out one sample from the thermostat for filtration at a time.

4.2 Take a sample of the dipeptide solution from each glass vials with a syringe (through a syringe needle). Remove the needle from the syringe and put on the syringe filter (see **Table of Materials**) to filter the dipeptide solution into the glass vial. Repeat the filtration with samples of other concentrations.

Analyze the filtrate ~~using an appropriate analytical method~~ in accordance with section 5.

NOTE: Refrain from centrifugation of samples as it takes up to a few minutes and may cause a change in the concentration equilibrium.

5 Derivatization and HPLC Analysis

5.1. Prepare the trifluoroacetic acid (TFA) solution.

Make the 50 mL solution of TFA in acetonitrile. Spike 0.34 mL of TFA in the measuring cylinder and adjust the volume of solution to 50 mL with acetonitrile at the room temperature.

CAUTION: Work with TFA under a fume hood with exhaust ventilation as trifluoroacetic acid is harmful when inhaled, causes severe skin burns and is toxic for aquatic organisms even at low concentrations[53].

5.2 Prepare the derivatization solution (Edman reagent[54])

Place 299 µL of phenyl isothiocyanate and 347 µL of triethylamine in the measuring cylinder and adjust the volume of solution to 50 mL with acetonitrile at the room temperature.

5.3. Sample derivatization

Prior to the high-performance liquid chromatography (HPLC) analysis derivatize the samples with Edman's reagent in the chromatography vials. Mix the 400 µL of the sample with 400 µL of the Edman's reagent. Heat the sample at 60 °C for 15 minutes. After heating, neutralize the sample with 225 µL of the solution of TFA and wait for a few minutes to cool sample to room temperature.

5.4. Analyze the sample using an appropriate method

Use HPLC (see **Table of Materials**) analysis to determine the concentration of the dipeptide solution before and after adsorption. Place the chromatography vials with the analyzed solutions into the HPLC autosampler and start analyzing samples with the necessary conditions, which are

311 set by the software (see **Table of Materials**).
312
313 NOTE: Mobile phase consists of 0.1% TFA in deionized water and pure acetonitrile, with
314 acetonitrile gradient from 20 to 90% at 286 nm for in 13 min. Analyze each sample in triplicate.
315 Measure the dipeptide solution concentration using the prior established calibration curve
316 (**Figure 2**). For chromatography specs see publication[55].
317

REPRESENTATIVE RESULTS:

Adsorption of dipeptide on nanocrystalline titanium dioxide was studied at the biocompatible conditions corresponding to the temperature range of 0−40 °C. Experimental dipeptide adsorption (A, mmol/g) on the surface of a titanium dioxide was evaluated as

$$A = \frac{V(C_0 - C_e)}{m} \qquad (1),$$

Where C_0 and C_e are the dipeptide starting and equilibrium concentrations, mM, respectively; V is the volume of a dipeptide solution of, L; and m is the weight of the sorbent, g.

The measurements of dipeptide adsorption were data processed using the Henry model. This isotherm model assumes the adsorption at relatively low concentrations with the sorbate molecules isolated from each other on a sorbent surface and it is suitable for describing the experimental data (**Figure 3**). Note, however, that this model can only be applied in the case of the reversible adsorbtion, which should be confirmed additionally. We suggest IR-spectroscopy of the mutltiple times rinsed material. The obtained equilibrium peptide amounts on the TiO_2 and solution are related in accordance with the linear equation:

$$A = K_H C_e \qquad (2)$$

where K_H is Henry's adsorption constant,

The equilibrium binding constant K_H was obtained from the slope of the dependence of dipeptide adsorption (A) on the dipeptide equilibrium concentration (C_e). The standard Gibbs free energy (ΔG, kJ/mol) for each temperature T was determined through the Van't Hoff equation:

$$\Delta G = -RT \ln K_H \qquad (3)$$

where R is the ideal gas constant, J/mol*K and T is the temperature of the adsorption process, K.

Dipeptide Gibbs free energies determined at each temperature (**Figure 4**) disclosed enthalpy (ΔH) as an interception of the linear regression with the ΔG axis. Regression variable – the entropy of the process (ΔS) was derived from the fundamental equation:

$$\Delta G = \Delta H - T\Delta S \qquad (4)$$

The calculated values of the equilibrium binding constant (K_H), standard Gibbs energy (ΔG), enthalpy (ΔH), and entropy (ΔS) for Ile-His are presented in **Table 1**.

FIGURE AND TABLE LEGENDS:

Figure 1: Dilution of 16 mM dipeptide stock solution.

Figure 2: Calibration curve at the different dipeptide concentration. (The dipeptide concentrations between 0.4 and 16.0 mM).

Figure 3: The dipeptide adsorption isotherms calculated by the Henry model for each temperature. (A), (B), (C), (D), (E) The dipeptide adsorption isotherm at 0 °C, 10 °C, 20 °C, 30 °C, 40 °C, respectively. (The calculated correlation coefficients R^2 fall into 0.96−0.99 range for all obtained Henry model isotherms). Error bars represent the 95% confidence interval for each sample concentarion measured in triplicate.

Figure 4: Dependence of the standard Gibbs free energy of the dipeptide adsorption on temperature~~s~~. Error bars represent the 95% confidence interval for Gibbs free energy as indirect measurement based on Henry Model.

Table 1: Thermodynamic Parameters of Dipeptide Adsorption.

DISCUSSION:

Adsorption from solutions for isotherm construction requires a longer time for equilibration due to kinetic restrictions and sorbents with the high specific surface area. Moreover, instability of soles, nanoparticle aggregates, crystallinity, nanoparticle size distribution, pH of the solution, competing for adsorption, etc. should be considered while adsorbing amino acids. And yet adsorption isotherm construction using depletion method remains to be the most available methodology that does not require expensive set-ups, providing exhaustive physical chemistry data for literally every soluble sorbate.

A distinction has to be made betwen adsorption modes: on solution dispersed particles and on fixed surface, when crystalline material is used as a sorbent. One should expect substantial difference in distribution of crystalline faces on macroscopically flat surfaces and on particles. Resulting thermodynamic parameters determined from adsorption of peptides on nanoparticle may not correspond to the thermodynamic parameters of peptide adsorption to macroscopically flat surfaces.

The average amount of peptides adsorbed on the inorganic surfaces is extremely low. At room temperature this value is about several hundreds of micrograms per square meter[28]. This small amount of adsorbate requires accurate measurement methods and solids with well-developed surfaces. Therefore, small-particle substances with a large specific surface (hundreds of square meters) should be used for adsorption experiments.[43,56–60]

Peptides are as proteins, a subtle matter, which retains its functionality at a narrow range of

conditions. In our experiments, we conducted adsorption experiments on nanocrystalline titanium dioxide at biocompatible temperatures: 0÷40 °C (273.15÷313.15 K) which relate to the normal functioning of a living organism. Adsorption at higher or lower temperatures are irrelevant and should not be considered for the experiment.

Multifunctional biologically active compounds also exhibit high susceptibility to pH of the media, as it affects the surface charge and therefore Coulomb interaction between charged functional groups[61–63]. Sorbent charge of oxide materials is also pH-dependent due to active proton exchange at the hydrated surface[64]. In order to establish pH stable condition for adsorption equilibrium use of a buffer is strongly required. In our study, we chose MES-buffer for its non-coordinating property[65], so it would not compete with peptide for adsorption on metal oxide surface like phosphate buffer[66].

Our recent modeling of adsorption of amino acids shows that major binding site on nanoparticle is the surface defect.[55] Defect distribution on the surface is one of the least controllable features of the nanocrystalline substrates, hence one should use sorbent from the same batch in order to maintain consistency in adsorption studies.

QCM, Plasmon resonance, ITC, are genuine methods with subtle sensitivity that in a combination of spectroscopic methods reveal structural peculiarities of the adsorbate during interplay with the surface. However, they do not overcome kinetic restrictions and still require considerable time to achieve adsorption equilibration. Furthermore, only one sample at a time can be processed, which makes batch sample analysis challenging. On the other hand, the depletion method is simple and only restricted with the thermostat capacity, making the processing of a large number of samples available to everyone.

Filtration of the thermostated samples should be accomplished as soon as samples have been evacuated from the thermostat in order to avoid temperature-induced reequilibration. Although equilibration at a new temperature may take up to a few hours, keeping adsorption samples at different temperature should be minimized. We also recommend refraining from centrifugation of samples for supernatant separation as it takes up to a few minutes and may cause a change in the concentration equilibrium. Choice of the filter material depend on the sorbate nature and should reduce possible filter-binding for the maximum recovery. We recommend following the vendor instructions on the choice of the specific filters.

Additionally, one should bear in mind that concentration change at the adsorption studies should be monitored using validated quantification method using mass-spectrometry, radio-spectroscopy or UV-Vis spectroscopy. The analysis is easy in case if adsorbate is spectroscopically active, otherwise additional labeling or derivatization of the adsorbate is required.

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The authors have nothing to disclose.

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