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SULPHONATE-TERMINATED CARBOSILANE DENDRONS COATED NANOTUBES: A GREENER POINT OF VIEW IN PROTEIN SAMPLE PREPARATION

Estefanía González-García[1], Carlos E. Gutiérrez Ulloa[2,3], Francisco Javier de la Mata[2,3], María Luisa Marina[1], María Concepción García[1*]

[1] Departamento de Química Analítica, Química Física e Ingeniería Química, Universidad de Alcalá, Ctra. Madrid-Barcelona Km. 33.600, 28871 Alcalá de Henares (Madrid), Spain.

[2] Departamento de Química Orgánica y Química Inorgánica, Universidad de Alcalá, Ctra. Madrid-Barcelona Km. 33.600, 28871 Alcalá de Henares (Madrid), Spain.

[3]Networking Research Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Spain.

[*]Corresponding author (e-mail: concepcion.garcia@uah.es; telephone + 34-91-8854915; fax + 34-91-8854971).

ABSTRACT

Reduction or removal of solvents and reagents in protein sample preparation is a demand. Dendrimers can strongly interact with proteins and have a great potential as a greener alternative to conventional methods used in protein sample preparation. This work proposes the use of single-walled carbon nanotubes (SWCNTs) functionalized with carbosilane dendrons with sulphonate groups for protein sample preparation and shows the successful application of the proposed methodology to extract proteins from a complex matrix. SEM images of nanotubes and mixtures of nanotubes and proteins were taken. Moreover, intrinsic fluorescence intensity of proteins was monitored observing most significant interactions at increasing dendron generations under neutral and basic pHs. Different conditions for the disruption of interactions between proteins and nanotubes after protein extraction and different concentrations of the disrupting reagent and the nanotube were also tried. Compatibility of extraction and disrupting conditions with the enzymatic digestion of proteins for obtaining bioactive peptides was also studied. Finally, sulphonate-terminated carbosilane dendrons coated SWCNTs enabled the extraction of proteins from a complex sample without using non-environmentally friendly solvents that were required so far.

Keywords: single-walled carbon nanotubes; sulphonate carbosilane dendrons; dendron coated nanotubes; protein-dendron interaction; protein extraction

INTRODUCTION

An aim in green chemistry philosophy is to develop and promote the use of procedures that limit the use of hazardous substances. Within the analytical chemistry background, green chemistry addressed the search for clean methodologies as alternative to polluting ones [1] which translates into the reduction or removal of solvents required in the different steps of the analytical process.

Protein sample preparation comprises all steps aimed to extract, purify, hydrolyze, fractionate, etc. proteins. Protein sample preparation is an important issue in many fields such as biotechnology, biochemistry or analytical chemistry. Within analytical chemistry, protein sample preparation is needed if we want to obtain bioactive peptides, to quantify proteins in a sample, to obtain a protein profile of a sample either using chromatographic or electrophoretic techniques, to study protein properties, to study interactions with other molecules or proteins, to assess food quality, to control the presence of allergens,to perform proteome analysis, etc. Most usual methods for protein extraction are trichloroacetic acid (TCA)-acetone and phenol-based extractions [2, 3], both using high amounts of environmentally adverse organic solvents. The development of new nanomaterials offers promising approaches to overcome the challenges in green protein sample preparation enabling to reduce the need for non-environmentally friendly solvents.

Dendrimers are nanostructured hyperbranched macromolecules with a structure composed by a core surrounded by branches and a multivalent external surface that can be easily functionalized [4, 5]. These characteristics have enabled dendrimers to be applied in diagnosis, treatment of different diseases, genes and drug delivery, molecular recognition, development of chemical sensors, enzymatic catalysis, etc [4, 5]. Phosphorous dendrimers, polylysine dendrimers, polypropylenimine dendrimers and

polyamidoaminedendrimers (PAMAM) are some examples of commercially available dendrimers that have been widely studied. Main focus now is to design new dendrimers with improved characteristics to be used in new applications.

Non-commercial carbosilane dendrimers present a strong silicon-carbon skeleton that confers them extraordinary properties such as a high thermodynamic, kinetic and hydrolytic stability, and a high biocompatibility and solubility in aqueous solutions [6, 7]. Moreover, due to their solubility in water, no expensive, hazardous or pollutant solvents are required for their use. Interactions between sulphonate-[8] or carboxylate-terminated carbosilane dendrimers [9] and proteins have been demonstrated. Carboxylate-terminated carbosilane dendrimers at acid pHs interacted with proteins resulting in their precipitation and separation from other substances in the solution. Carboxylate-terminated carbosilane dendrimers have been employed in protein sample preparation and purposed as a more sustainable and cheaper alternative than usual methods. Unlike them, sulphonated-terminated dendrimers interacting with proteins remain in solution together with other substances that do not interact with the dendrimer. This fact limits the application of these dendrimers in protein sample preparation despite their strong interaction with proteins.

Single-walled carbon nanotubes (SWCNTs) are allotropes of carbon with a hollow cylindrical structure of a unique graphene sheet. They possess an extraordinary combination of mechanical and optical properties and electrical and thermal conductivities, as well as a large surface area. These characteristics have made them suitable for their use in a wide range of applications [10-12]. Many of these applications are based on interactions between proteins and SWCNTs [13-17]. Moreover, functionalization of nanotubes could enhance their properties and interactions with other molecules, improving their applications, e.g. in drug delivery and targeting [18].

SWCNTs functionalized with dendritic systems have been applied for the production of biodiesel [19], for the detection of molecules related to acetaminophen toxicity treatment [20], as anti-scavenger during unsaturated polyester curing process [21], for the development of biosensors [22], for selective detections, etc [23, 24]. In these works, commercial PAMAM was the main dendrimer employed for coating SWCNTs [25-28]. In this regard, carbosilane dendrimers have never been employed to coat SWCNTs.

On the other hand, the development of methodologies for the explotation of food industry byproducts and the use of sustainable resources is also a hot topic within green philosophy. In this line, previous works have demonstrated that fruit stones are sustainable sources of proteins and bioactive peptides [29-31]. Nevertheless, proposed methodology to extract proteins from fruit stones involves expensive and polluting solvents and reagents. The aim of the present work was to study the potential of sulphonate-terminated carbosilane dendrons coated SWCNTs to extract proteins evaluating the effect of pH, dendron generations, protein nature on the established interaction. Conditions promoting interactions were applied to the extraction of proteins from fruit stones studying the compatibility of these conditions with protein hydrolysis.

EXPERIMENTAL SECTION

Reagents and materials. All chemical and reagents were of analytical grade. Water was daily obtained from a Milli-Q system from Millipore (Bedford, MA, USA). Tris(hydroxymethyl)aminomethane (Tris), hydrochloric acid (HCl), sodium dodecyl sulphate (SDS), and sodium chloride (NaCl) were acquired at Merck (Darmstadt, Germany). Acetic acid (AA), methanol (MeOH), and hexane were from ScharlauChemie(Barcelona, Spain). Dithiothreitol (DTT), β-mercaptoethanol (β-ME), o-phthalaldehyde (OPA), sodium tetraborate, glutathione (GSH), sodium hydroxide, trifluoroacetic acid (TFA), bovine serum albumin (BSA), lysozyme from chicken egg white, and myoglobin from equine heart were purchased in Sigma-Aldrich (Saint Louis, MO, USA). Laemmli buffer, Mini-Protean precast gels, Tris/glycine/SDS running buffer, Precision Plus Protein Standards (recombinant proteins expressed by *E. Coli* with molecular weights of 10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa), Bio-Safe Coomassie stain, and Bradford reagent (Coomassie Blue G-250) were acquired at Bio-Rad (Hercules, CA, USA). Alcalase 2.4 L FG was kindly donated by Novozymes Spain S.A. (Madrid, Spain). Unfunctionalized single-walled carbon nanotubes (U-SWCNT) (diameter of 1.5 nm and length of 1-5 μm) were from NanoLab (Waltham, MA, USA). Plums were purchased in a local market (Alcalá de Henares, Madrid, Spain).

Sulphonate-terminated carbosilane dendrons coated carbon nanotubes. SWCNTs were oxidized according to the method of Lakshminarayanan et al [32]. and subsequently dendronized using carbosilane dendrons of first to third generation functionalized with a pyrene unit at the focal point and sulphonate groups at the periphery, through π-π interactions, leading to the formation of hybrid systems: 1G-SWCNT, 2G-SWCNT, and 3G-SWCNT (**Fig. 1**). A scheme of the interaction between carbosilane dendrons of first, second and third generation, containing pyrene at the focal

point and sulphonate groups at the periphery, with single-wall CNTs is showed in the supplementary material (**Figure S1**).

Scanning electron microscopy (SEM). A Zeiss DSM-950 (*Digital Scanning Microscope*) (Carl Zeiss, Obercochen, Germany) at 30 kV was employed. Solutions of 0.03 μM of every protein containing U-SWCNT or SWCNT-3G (at a dendron concentration of 0.2 μM) were prepared in water, homogenized, and lyophilized. Fine powders of these mixtures were deposited on an aluminum SEM sample stub and gold-coated using a Polaron E5400 sputter coater in order to intensify the electrical conductivity of the surface and, thus, to improve the optical resolution. Images of controls of U-SWCNTs and SWCNTs-3G without protein were also taken.

Fluorescence measurements. Fluorescence intensity was measured in a RF-1501 spectrofluorometer from Shimadzu (Kioto, Japan). $\lambda_{exc}$was fixed at 279-281 nm and emission was recorded at λ_{em} ranging from 290 to 400 nm. Six nanotube-protein solutions with different dendron concentrations (0.005, 0.01, 0.05, 0.1, and 0.2 μM) and constant protein concentration (0.03 μM) were prepared in water (pH 7.5), 0.1% TFA (pH 1.8) and 5 mM Tris-HCl buffer (pH 9.0). Fluorescence of nanotubes and dendron-nanotubes blanks was measured at every pH in absence of proteins. Three measurements of each two replicates were made. Proteins fluorescence quenching was studied through Stern-Volmer model using the following equation:

$$I_0/I = 1 + K_{SV}[Q]$$

where I is the fluorescence intensity of proteins at the different dendron concentrations, I_0 is the fluorescence intensity of proteins in absence of dendron, K_{SV} is the Stern-Volmer constant, and $[Q]$ is the dendron concentration. Data were expressed as mean ± standard deviation of 6 measurements corresponding to two independent samples measured in triplicate.

Vegetable sample protein extraction and analysis. Plum seed proteins extraction was made following the method of González-García et al. [29] Briefly, plum stones were crashed with a nutcracker to obtain the seed that was milled and defatted with hexane. Next, 30 mg of defatted seeds were extracted using four different procedures (I-IV). Procedure I used just water; procedure II used a buffered solution (100 mM Tris-HCl (pH 7.5) + 1 % (w/v) SDS + 0.5 % (w/v) DTT); procedure III employed U-SWCNTs, and procedure IV used SWCNTs-3G. In procedures I-III, solutions were extracted using a high intensity focused ultrasound (HIFU) probe (model VCX130, Sonics Vibra-Cell, Hartford, CT, USA) at 30 % of amplitude for 1 min. In procedure IV, solutions were shaken for 1 h since ultrasounds could disrupt dendron-nanotube interaction. Extracted proteins were centrifuged and supernatants were collected. Supernatants were filtered through Amicon Ultra-4 molecular weight cut off (MWCO) filters of 100 kDa for 90 min at 4,000g to separate extracted proteins from other molecules including SWCNT (in the case of procedures III and IV). In procedures I and II, the extracts were purified by precipitation with acetone (ratio 1:2) and centrifuged for 10 min at 4,000g and pellets were reconstituted in their corresponding media. In procedures III and IV, the interaction between extracted proteins and SWCNT was disrupted and proteins separated from SWCNT by ultrafiltration. Four different solutions to disrupt interactions were tried: (S1) water at 25 ºC, (S2) water at 50 ºC, (S3) 0.1% (w/v) SDS, and (S3) 1M NaCl. After the second ultrafiltration, recovered solutions (theoretically, just proteins) were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). All procedures are summarized in the scheme of **Fig. 2**.

Protein separation by SDS-PAGE was performed by mixing 15 µL of sample solution with 15 µL of Laemmli buffer containing 5% (v/v) β-ME and heating during 5 min at 100 ºC. Electrophoresis was carried out on a Bio-Rad Mini-Protean system employing

Ready Precast Gels and using Tris/glycine/SDS as running buffer. After protein separation, they were fixed using a mixture of H_2O/MeOH/AA (50:40:10) for 30 min, stained with Bio-Safe Coomassie stain (50 mL) for 2 h, and washed with water for another 2 h. Protein content in solutions was estimated by Bradford assay [33]. Two independent replicates of each procedure were measured in triplicate.

Protein digestion. Plum seed protein extracts obtained with the four different procedures were hydrolyzed employing Alcalase enzyme. For that purpose, enzyme was added to every extract at a concentration of 0.3 AU/g protein and the mixtures were incubated in a Thermomixer Compact (Eppendorf AG, Hamburg, Germany) during 3 h at 50 ºC with agitation. Finally, temperature was raised to 100 ºC for 10 min to stop the reaction and hydrolysates were centrifuged for 10 min at 7,000g. Supernatants were collected and peptide concentration was determined by OPA assay using the method described by González-García *et al.* [29] The degree of hydrolysis (% DH) was calculated by the following equation:

$$DH\ (\%) = \frac{peptide\ content}{protein\ content}\ x\ 100$$

RESULTS AND DISCUSSION

The evaluation of potential interactions between proteins and nanotubes modified with dendrons was carried out by monitoring the protein intrinsic fluorescence intensity and its maximum emission wavelength. For that purpose, three standard proteins with different molecular weights and isoelectric points were employed: BSA (66.5 kDa and a pI of 4.7), lysozyme (14.3 kDa and a pI of 11.35), and myoglobin (17.8 kDa and a pI of 6.8). SWCNTs functionalized with sulphonate-terminated dendrons from first to third generation (1G, 2G, and 3G) were used (1G-SWCNT, 2G-SWCNT, and 3G-SWCNT). Increasing dendron generations supposed the increase of sulphonate groups from 2 (1G-SWCNT) to 8 (3G-SWCNT), which, due to their low pKa, remained deprotonated at all pHs. Scanning Electron Microscopy was employed for a first characterization of samples. As example, **Fig. 3** showsSEM images corresponding to the three proteins in presence of U-SWCNTs and 3G-SWCNTs. These images demonstrated that the presence of proteins induced changes in SEM images of nanotubes. In fact, nanotubes in absence of proteins showed a compacted structure while they looked like more dispersed in presence of proteins. Moreover, samples containing proteins showed sheet structures that could correspond to proteins [34]. In order to demonstrate that these changes could be due to interactions, protein fluorescence studies were next carried out.

Fluorescence study of interactions between proteins and dendron coated nanotubes. Tryptophan (Trp) residues in proteins are the main responsible of proteins fluorescence and any disturbance in the surrounding of Trp residues could promote a change in the protein fluorescence intensity and/or emission wavelength [35, 36].

The variation of BSA fluorescence intensity when adding increasing concentrations (0.005 – 0.2 μM) of the three functionalized nanotubes (1G-SWCNTs,

206 2G-SWCNTs, and 3G-SWCNTs) and the unfunctionalized nanotubes, at all tested pHs,
207 is shown in **Fig. 4a**. Regardless of the functionalization of the nanotube, the dendron
208 generation or the pH, initial BSA fluorescence intensity decreased along with the
209 SWCNT concentration. This fluorescence quenching did not come along with an
210 emission wavelength variation. Moreover, the fluorescence intensity decrease was less
211 significant for the unfunctionalized nanotube and greater for the nanotubes
212 functionalized with third generation dendrons. Additionally, this decrease was greater at
213 neutral pH and lower at more extreme pHs. The decrease in fluorescence intensity is
214 due to a change in the protein structure that has resulted in a less exposition of the two
215 BSA Trp residues: Trp-134, located in the surface of the molecule, and Trp-213, placed
216 in a hydrophobic cavity [37]. The change in protein structure could be due to a protein
217 unfolding, to the deactivation of the excited protein by collision, to the formation of a
218 complex, etc. In order to further study the cause of this decrease in the fluorescence
219 intensity, the ratio (I_0/I) was plotted against the nanotubes concentration by application
220 of the Stern-Volmer equation (see **Fig. 4b**). A linear relationship was observed in all
221 cases and the values of slopes were grouped in **Table 1**. These slopes inform about the
222 accessibility of Trp residues to the nanotube. As expected, higher slopes were observed
223 with dendron coated nanotubes than with unfunctionalized nanotubes. Within
224 functionalized nanotubes, higher slopes were obtained at increasing dendron
225 generations. This feature is in agreement with previous researches performed with
226 sulphonate-terminated carbosilane dendrimers and it could be related to the higher
227 number of functional groups in higher dendrons generation [8]. Nevertheless, unlike
228 that research in which stronger interactions were observed at acidic pH, nanotubes
229 showed the highest slopes at neutral pH, where both BSA and dendron were negatively
230 charged. The interaction at neutral pH could be due to electrostatic forces since, despite

231 the overall negative charge of BSA, modeling [8] showed that there were local cationic
232 sites that, from an electrostatic point of view, could bind sulphonate groups. Moreover,
233 other non-electrostatic interactions such as hydrophobic, π-π stacking or Van der Waals
234 could also contribute to a blocking of Trp residues and a decrease in fluorescence
235 intensity. Differences with results obtained with sulphonate-terminated carbosilane
236 dendrimers could be justified by the differences in structure and size between
237 dendrimers and dendrons. Moreover, in comparison with sulphonate-terminated
238 dendrimers, the slopes obtained with coated nanotubes were much lower. This could be
239 explained taking into account the higher amount of charges of dendrimers (1G (8
240 functional groups), 2G (16 functional groups), and 3G (32 functional groups)) in
241 comparison with dendrons (1G-SWCNT (2 functional groups), 2G-SWCNT (4
242 functional groups), and 3G-SWCNT (8 functional groups)) and the higher size and
243 steric impediment in SWCNTs.

244 Similar fluorescence variations were obtained with lysozyme (see **Fig. 5**). As for
245 BSA, no shift in the emission wavelength was observed. Stern-Volmer slopes were also
246 calculated and values were grouped in **Table 1**. As expected, higher slopes were
247 obtained for the coated nanotubes and for the third generation dendron. Lysozyme
248 showed the highest slopes at neutral and basic pHs. Again, the slopes obtained for
249 lysozyme in presence of sulphonate-terminated carbosilane dendrons coated nanotubes
250 were much lower than the obtained with sulphonate-terminated carbosilane dendrimers
251 In comparison with previous results obtained with BSA, in general, Stern-Volmer [8]
252 slopes were higher for lysozyme. This fact could be justified by the higher number of
253 Trp residues of lysozyme, the net positive charge that this protein shows at all tested
254 pHs, and the smaller size of the protein that could access more easily to sulphonate
255 groups.

Fig. 6a shows the effect of the addition of nanotubes at different pHs to a myoglobin solution. This protein presents a *heme* group that is attached to the protein at neutral and basic pHs [38]. The presence of SWCNTs resulted in a decrease in the protein intrinsic fluorescence intensity at acidic pH. This behavior was more significant for coated nanotubes and for higher dendron generations. Nevertheless, this tendency was not observed at neutral and basic pHs, unlike BSA and lysozyme. Indeed, at these pHs, the fluorescence intensity was kept when increasing the nanotube concentration, regardless it was functionalized or not. This result could suggest that myoglobin, at neutral and basic pHs, was not interacting with the nanotube or that the interactions did not produce a modification in the surrounding of Trp residues. The singular behavior of myoglobin was also observed previously [8, 9] and it could be related to the *heme* group that is attached to the protein at these pHs. Again, no significant emission wavelength variation was appreciated. **Fig. 6b** shows the Stern-Volmer plot obtained for myoglobin at acidic pH and **Table 1** groups the slopes obtained. Like BSA and lysozyme, the Stern-Volmer slope was higher for the coated nanotubes and for the 3G-SWCNT. These slopes were much lower than the observed with sulphonate-terminated carbosilane dendrimers that, as with BSA and lysozyme, could be related to the different structure of dendrimers and dendrons. Moreover, these slopes were very similar to the previously observed for lysozyme and higher than the observed for BSA. This fact could be explained by the lower size of lysozyme (14.3 kDa) and myoglobin (17.8 kDa) and the higher size of BSA (66.5 kDa) that could result in more significant steric impediments. The interaction between myoglobin and SWCNTs at acidic pH could be of electrostatic nature considering their opposite charge.

Application of nanotubes to protein sample preparation. Once demonstrated the interaction between dendron coated nanotubes and proteins, they were employed for the

281 extraction of proteins from a complex sample. As explain in the introduction,
282 exploitation of food byproducts with high protein contents by the extraction of proteins
283 requires the development of green methods to make sense to the use of this sustainable
284 resources. It would be nonsense to try to recover these proteins using non-sustainable
285 methodologies. Four different procedures were employed (Procedures I-IV) for the
286 extraction of proteins from the fruit (plum) stone. Proteins from plum seeds were
287 extracted with water (Procedure I) and following a protocol previously optimized for the
288 quantitative extraction of proteins from plum seeds (Procedure II) [29]. This protocol
289 involved the extraction with a 100 mM Tris-HCl buffer (pH 7.5) containing 1 % (w/v)
290 SDS and 0.5 % (w/v) DTT. In Procedures I and II, a clean-up step by precipitation of
291 proteins with acetone was also conducted. Results were compared with the obtained
292 when using U-SWCNTs (Procedure III) and 3G-SWCNTs (Procedure IV) solutions at
293 pH 7.5 at a concentration of 2µM. This concentration, higher than the used in the
294 evaluation of interactions between nanotubes and model proteins, was chosen taking
295 into account the high protein content of plum seeds. In all cases, after centrifugation,
296 supernatants were ultrafiltrated through 100 kDa cut-off filters. This step was not
297 needed in the case of Protocol I and II, since there is a final clean up by acetone
298 precipitation, but it was included in these experiments to ensure that differences in
299 protein yield were not due to the possible retention of proteins in the ultrafiltration filter.
300 Next, all solutions were collected and analyzed by SDS-PAGE.

301 **Fig. 7** shows the electrophoretic profiles obtained by SDS-PAGE. Moreover, the
302 protein yield obtained with every procedure (related to the highest protein yield) and
303 determined by Bradford assay is also included in this figure. In all cases, bands below
304 75 kDa with two significant bands around 20 kDa were obtained. More intense bands
305 were observed when extracting under optimized buffered conditions (line 2). Protein

306 contents in extracts confirmed this fact. When extracts were treated with U-SWCNTs
307 and 3G-SWCNTs, proteins interacted with SWCNTs forming a complex that did not
308 pass through the 100 kDa cut-off filter (see lines 3 and 8, respectively). In order to
309 disrupt protein-nanotube interactions after the extraction, different reagents were tried:
310 water at room temperature, water at 50 ºC, 0.1% (w/v) SDS, and 1M NaCl. The use of
311 water at room temperature, both with U-SWCNTs and 3G-SWCNTs (lines 4 and 9), did
312 not disrupt the interaction and no band or signal was observed by SDS-PAGE or
313 Bradford assay. The use of water at 50 ºC (lines 5 and 10) resulted in very low protein
314 recoveries while the use of SDS (lines 6 and 11) or NaCl (lines 7 and 12) resulted in
315 higher protein recoveries (higher than 70% for U-SWCNTs and higher than 85% for
316 3G-SWCNTs). Thus, the best extraction conditions would be the Procedure IV using
317 3G-SWCNTs and SDS or NaCl as disrupting reagents. Moreover, in all cases a high
318 reproducibility in obtained protein yields was observed.

319 Finally, and in order to demonstrate the effect of the ultrafiltration step on the
320 recovery of proteins, the conventional method (Protocol II) with and without the
321 ultrafiltration step was employed for the extraction of proteins from the fruit stones.
322 Results showed no significant differences between protein contents obtained in both
323 cases ($p > 0.01$) demonstrating that proteins did not remain retained or absorbed in this
324 filter.

325 **Effect of the extracting procedure on the enzymatic digestion of proteins.** Proteins
326 are usually extracted for their subsequent enzymatic digestion for identification
327 purposes or to obtain bioactive peptides. Therefore, a further investigation was
328 addressed to study the compatibility of Procedure IV with the enzymatic digestion of
329 the extracted proteins, employing for that a protease enabling the obtaining of bioactive
330 peptides. Indeed, in-solution digestion of proteins could be hindered by the presence of

reagents such as SDS, used in the disruption of protein-SWCNT interactions. In order to evaluate this fact, proteins extracted by Procedure IV using different disrupting conditions were digested with Alcalase enzyme and the degree of hydrolysis (DH, %) was determined by the OPA assay. Results are shown in **Table 2** along with the DHs obtained using the other tested procedures (Procedures I-III). Regardless of the extracting procedure, DH ranged from 65 to 70 %, except when SDS was used for the disruption of protein-nanotubes interactions in Procedures III and IV that showed DH from 25 to 27 %. Therefore, despite Procedure IV using 3G-SWCNTs and SDS as disrupting reagent seemed to be the best alternative to Procedure II (buffered extraction) in terms of protein recovery (87%), it is not a right option when proteins must be in-solution digested. In this case, extraction using Procedure IV and 1M NaCl as disrupting reagent could be a better alternative. However, protein yield in Procedure IV is not as high as the obtained by Procedure II (buffered extraction). In order to increase the recovery of proteins when using Procedure IV, the effect of the concentration of 3G-SWCNTs was evaluated (see **Table 3**). The initial nanotube concentration (2 μM) was increased up to 10 μM observing an increase in protein yield up to nanotube concentrations close to 8 μM. Above this value, no significant change was observed. Moreover, a study of the effect of the NaCl concentration on the recovery of proteins was also carried out. Four different NaCl concentrations in the range from 0.5 to 2.0 M were tried observing an increase in protein recovery when NaCl concentration increased from 0.5 M NaCl to 1 M NaCl. No further increase was obtained for higher NaCl concentrations. Therefore, final optimized conditions (extraction of proteins with 8 μM of 3G-SWCNT and disruption of interaction with 1 M NaCl) enabled a protein recovery of 97.1 ± 1.1 % with respect to the maximum recovery (100%) obtained with Procedure II (100 mM Tris-HCl buffer with 0.1% SDS and 0.5% DTT followed by acetone

356 precipitation). Statistical analysis showed that there was not significant differences
357 between protein yields obtained by the conventional procedure and the proposed one
358 using sulphonate-terminated carbosilane dendrons coated nanotubes ($p > 0.01$). These
359 results demonstrated that the extraction of proteins using dendron coated nanotubes is a
360 "clean", sustainable and cheap alternative to conventional methodologies usually
361 employed in protein sample preparation.

362

CONCLUSIONS

Sulphonate-terminated carbosilane dendrons coated nanotubes are a green alternative to usual methods employed in protein sample preparation and that require the use of organic solvents and other hazardous reagents. The interaction between proteins and sulphonate-terminated carbosilane dendrons coated nanotubes was demonstrated by SEM images and by the monitoring of the internal fluorescence of proteins mainly due to Trp residues. Protein-nanosystem interactions were promoted under neutral pH conditions and with the nanotube functionalized with the highest generation dendrons. The attachment of this nanostructure to the nanotube made possible its application in protein sample preparation in comparison with sulphonate-terminated carbosilane dendrimers. Functionalized nanotubes were successfully employed for the extraction of proteins from a complex sample. Recovery of proteins when extracting using functionalized nanotubes under optimized conditions (8 μM 3G-SWCNTs (pH 7.5) and 1 M NaCl for the disruption of interactions) was similar to the observed when using the conventional procedure and much higher than when using unfunctionalized nanotubes. Additionally, the extraction of proteins using 3G-SWCNT was compatible with in-solution protein digestion observing hydrolysis degrees similar to the obtained using the traditional procedure.

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FIGURE CAPTIONS

Fig. 1 Oxidation and dendronization of SWCNTs using anionic carbosilane dendron functionalized with pyrene at the focal point of third generation.

Fig. 2 Scheme followed in the extraction of proteins using four different procedures.

Fig. 3 Scanning electron microscopy (SEM) images of unfunctionalized single walled carbon nanotubes (U-SWCNTs) and the third generation of sulphonate-terminated carbosilane dendron coated nanotubes (3G-SWCNTs), alone and with the three different proteins (BSA, lysozyme (LYZ), and myoglobin (MYO)).

Fig. 4 Fluorescence intensity variation **(a)** and Stern-Volmer plots **(b)** corresponding to BSA solutions in presence of increasing concentrations of unfunctionalized single walled carbon nanotubes (U-SWCNTs) and sulphonate-terminated carbosilane dendron coated nanotubes (1G-SWCNTs, 2G-SWCNTs, and 3G-SWCNTs) at pHs 1.8, 7.5, and 9.0. Error bars show the standard deviation corresponding to two independent samples measured by triplicate. Concentrations in x-axis represent the dendron concentration. In the case of U-SWCNTs, concentrations are equivalent to the nanotube concentration needed for the preparation of functionalized ones.

Fig. 5 Fluorescence intensity variation **(a)** and Stern-Volmer plots **(b)** corresponding to lysozyme solutions in presence of increasing concentrations of unfunctionalized single walled carbon nanotubes (U-SWCNTs) and sulphonate-terminated carbosilane dendron coated nanotubes (1G-SWCNTs, 2G-SWCNTs, and 3G-SWCNTs) at pHs 1.8, 7.5, and 9.0. Error bars show the standard deviation corresponding to two independent samples measured by triplicate. Concentrations in x-axis represent the dendron concentration. In the case of U-SWCNTs, concentrations are equivalent to the nanotube concentration needed for the preparation of functionalized ones.

532 **Fig. 6** Fluorescence intensity variation **(a)** and Stern-Volmer plots **(b)** corresponding to
533 myoglobin solutions in presence of increasing concentrations of unfunctionalized single
534 walled carbon nanotubes (U-SWCNTs) and sulphonate-terminated carbosilane dendron
535 coated nanotubes (1G-SWCNTs, 2G-SWCNTs, and 3G-SWCNTs) at pHs 1.8, 7.5, and
536 9.0. Error bars show the standard deviation corresponding to two independent samples
537 measured by triplicate. Concentrations in x-axis represent the dendron concentration. In
538 the case of U-SWCNTs, concentrations are equivalent to the nanotube concentration
539 needed for the preparation of functionalized ones.

540 **Fig. 7** Electrophoretic profiles obtained by SDS-PAGE and protein yield (expressed as
541 mean ± standard deviation corresponding to three independent measurements of each
542 two replicates) corresponding to the extraction of proteins from plum seeds using
543 different procedures: lane 1, Procedure I; lane 2, Procedure II; lane 3, Procedure III with
544 no interaction disruption; lane 4, Procedure III and interaction disruption with water at
545 room temperature; lane 5, Procedure III and interaction disruption with water at 50 ºC;
546 lane 6, Procedure III and interaction disruption with 0.1 % (w/v) SDS; lane 7, Procedure
547 III and interaction disruption with 1 M NaCl; lane 8, Procedure IV with no interaction
548 disruption; lane 9, Procedure IV and interaction disruption with water at room
549 temperature; lane 10, Procedure IV and interaction disruption with water at 50 ºC; lane
550 11, Procedure IV and interaction disruption with 0.1 % (w/v) SDS; lane 12, Procedure
551 IV and interaction disruption with 1 M NaCl.

552

553 **Table 1.** Stern-Volmer constants (K_{SV}) calculated from the decreasing fluorescence
554 intensities of proteins observed when adding single-walled carbon nanotubes
555 (SWCNTs) at three different pHs (1.8, 7.5, and 9.0).[a]

Protein		K_{SV} ($M^{-1} \cdot 10^3$)		
		pH 1.8	**pH 7.5**	**pH 9.0**
BSA ***66.5 kDa*** ***pI = 4.7***	**Protein charge**[b]	+97	-17	-22
	U-SWCNT	0.97 ± 0.06	2.30 ± 0.06	0.31 ± 0.08
	1G-SWCNT	2.56 ± 0.07	9.63 ± 0.05	1.80 ± 0.08
	2G-SWCNT	3.52 ± 0.06	11.04 ± 0.05	2.19 ± 0.07
	3G-SWCNT	4.04 ± 0.06	11.06 ± 0.05	3.64 ± 0.07
Lysozyme ***14.3 kDa*** ***pI = 11.35***	**Protein charge**	+17	+8	+8
	U-SWCNT	1.36 ± 0.11	1.50 ± 0.05	2.30 ± 0.07
	1G-SWCNT	1.79 ± 0.08	4.79 ± 0.04	4.66 ± 0.06
	2G-SWCNT	7.32 ± 0.06	12.16 ± 0.04	10.78 ± 0.05
	3G-SWCNT	9.00 ± 0.07	17.07 ± 0.03	22.62 ± 0.04
Myoglobin ***17.8 kDa*** ***pI = 6.8***	**Protein charge**	+31	-2	-2
	U-SWCNT	1.04 ± 0.09	-	-
	1G-SWCNT	6.30 ± 0.07	-	-
	2G-SWCNT	7.59 ± 0.07	-	-
	3G-SWCNT	9.44 ± 0.09	-	-

556 [a] Data were expressed as mean ± standard deviation corresponding to two independent
557 samples measured by triplicate.

558 [b] Values were taken from reference [8].

559

560

561 **Table 2.** Degree of hydrolysis (%DH) obtained after digestion with Alcalase enzyme of
562 plum seed proteins extracted using different extraction procedures.

Extraction procedure	Disrupting solution	%DH
Procedure I: Extraction with water, centrifugation, ultrafiltration through 100 kDa MWCO filter, and precipitation of proteins with acetone		67.3 ± 3.5
Procedure II: Extraction with a 100 mM Tris-HCl (pH 7.5), 1% SDS and 0.5% DTT, centrifugation, ultrafiltration through 100 kDa MWCO filter and precipitation of proteins with acetone		69.9 ± 3.1
Extraction procedure	**Disrupting solution**	**%DH**
Procedure III: Extraction with U-SWCNTs (2µM, pH 7.5), ultrafiltration through 100 kDa MWCO filter, disruption of interaction with a selected solution, and ultrafiltration through 100 kDa MWCO filter	Water at room temperature	66.4 ± 5.1
	Water at 50 ºC	66.9 ± 4.3
	0.1% SDS	27.2 ± 3.9
	1 M NaCl	68.4 ± 2.7
Procedure IV: Extraction with 3G-SWCNTs (2µM, pH 7.5), ultrafiltration through 100 kDa MWCO filter, disruption of interaction with a selected solution, and ultrafiltration through 100 kDa MWCO filter solution	Water at room temperature	67.0 ± 3.9
	Water at 50 ºC	66.8 ± 5.0
	0.1% SDS	25.6 ± 4.8
	1 M NaCl	69.1 ± 3.7

563
564

565 **Table 3.** Protein recovery obtained using Procedure IV with different 3G-SWCNTs and

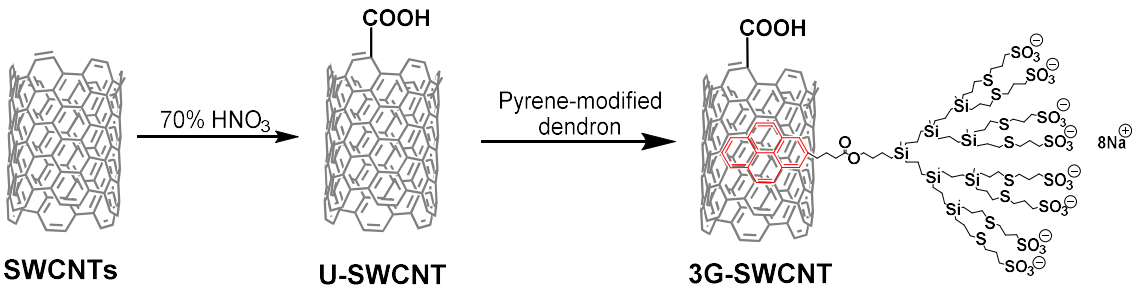
566 NaCl concentrations.

	Concentration	Protein recovery (%)
Variation of the concentration of 3G-SWCNTs[a]	2 μM	85.3 ± 0.9
	4 μM	88.9 ± 1.8
	6 μM	93.0 ± 1.5
	8 μM	96.7 ± 1.2
	10 μM	97.1 ± 1.1
Variation of the concentration of NaCl[b]	0.5 M	90.3 ± 1.9
	1.0 M	97.1 ± 1.1
	1.5 M	97.0 ± 1.5
	2.0 M	97.3 ± 1.6

567 [a] The NaCl concentration in these experiments was set to 1 M.

568 [b] The 3G-SWCNT concentration in these experiments was set to 8 μM.

569



570

571

572 **Fig. 1**

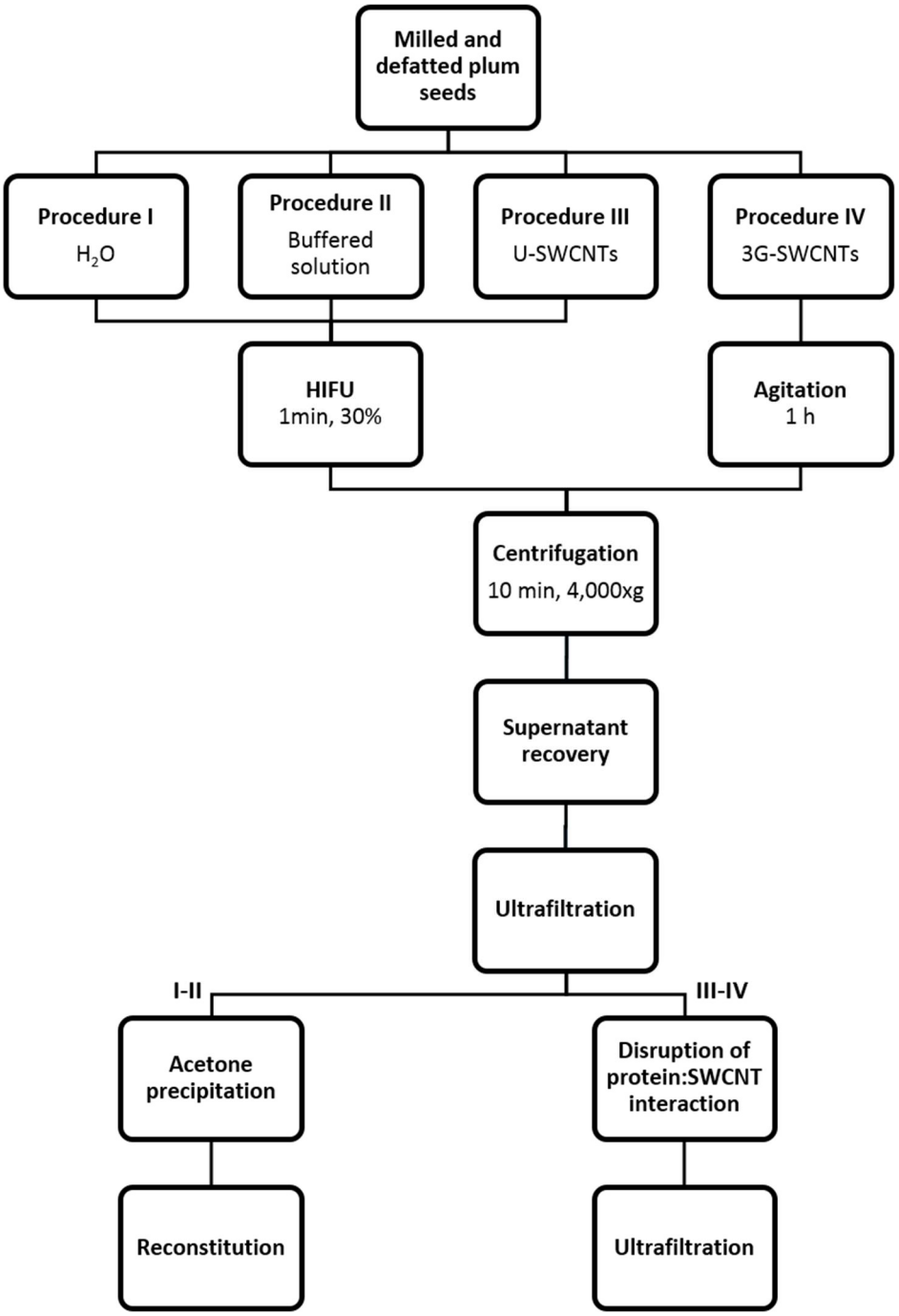

Milled and defatted plum seeds
Procedure I
H_2O
Procedure II
Buffered solution
Procedure III
U-SWCNTs
Procedure IV
3G-SWCNTs
HIFU
1min, 30%
Agitation
1 h
Centrifugation
10 min, 4,000xg
Supernatant recovery
Ultrafiltration
I-II
III-IV
Acetone precipitation
Disruption of protein:SWCNT interaction
Reconstitution
Ultrafiltration

Fig. 2

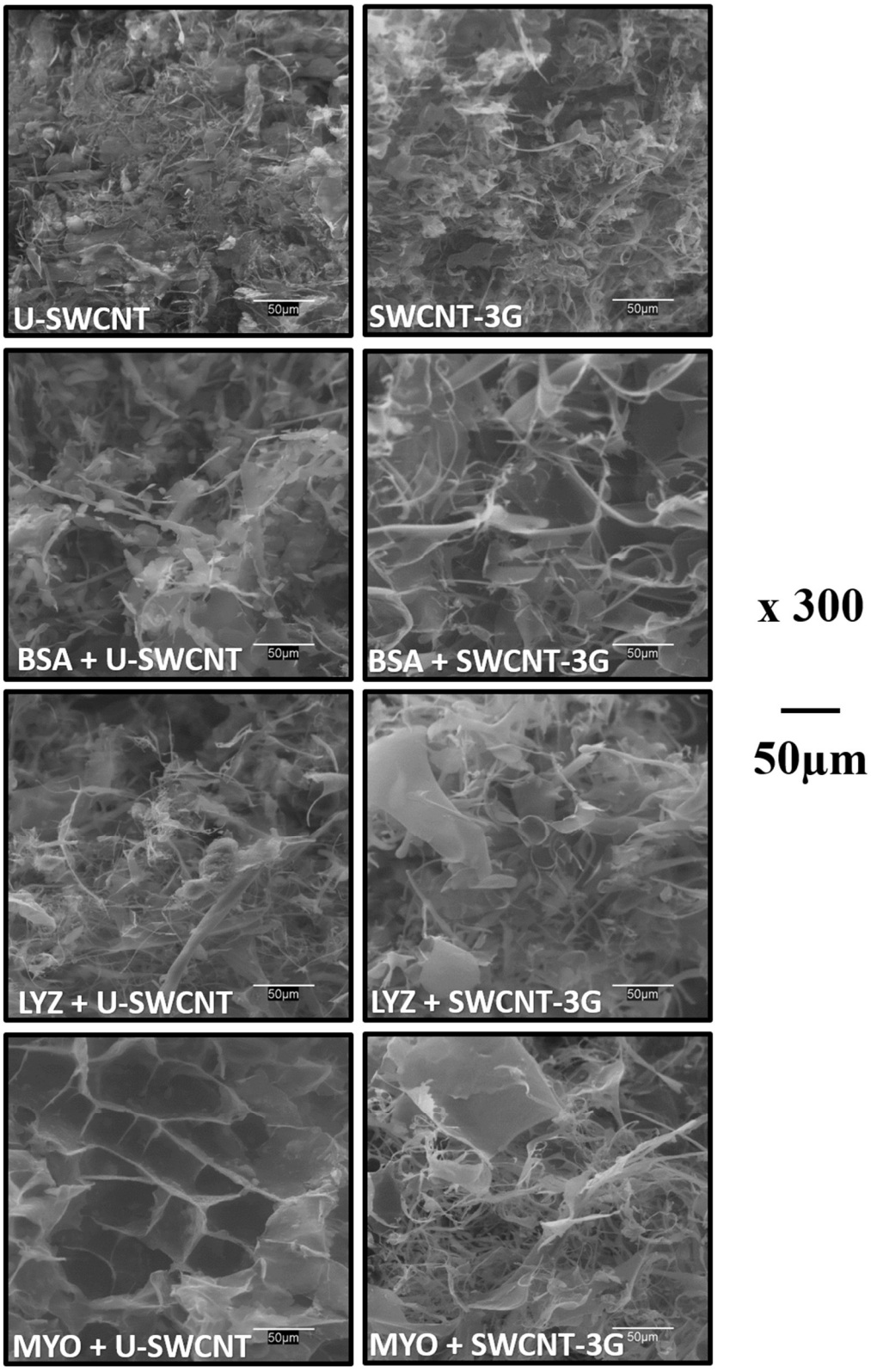

U-SWCNT
50µm
SWCNT-3G
50µm
BSA + U-SWCNT
50µm
BSA + SWCNT-3G
50µm
LYZ + U-SWCNT
50µm
LYZ + SWCNT-3G
50µm
MYO + U-SWCNT
50µm
MYO + SWCNT-3G
50µm
x 300
50µm

Fig. 3

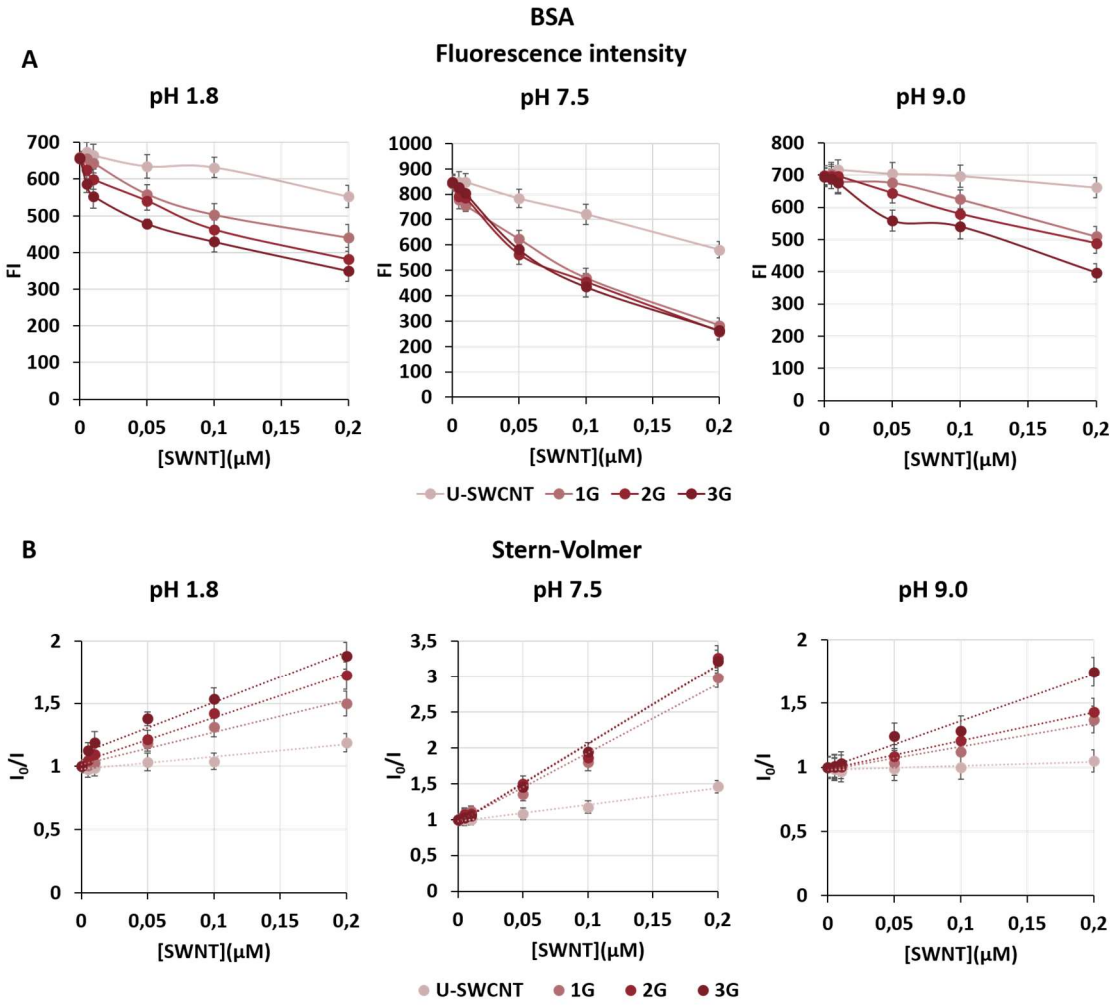

BSA
A
Fluorescence intensity
pH 1.8
pH 7.5
pH 9.0
FI
[SWNT](μM)
U-SWCNT
1G
2G
3G
B
Stern-Volmer
pH 1.8
pH 7.5
pH 9.0
I0/I
[SWNT](μM)
U-SWCNT
1G
2G
3G

Fig. 4

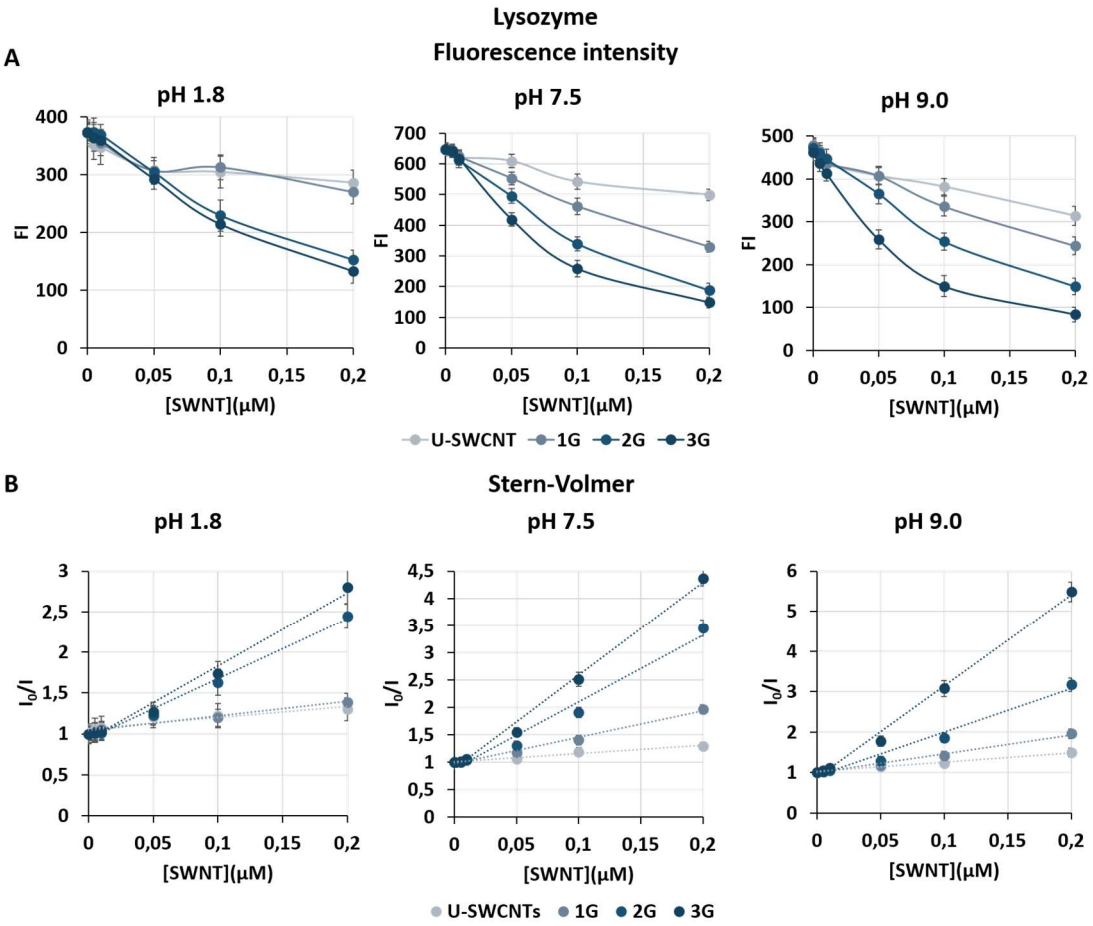

A
Lysozyme
Fluorescence intensity
pH 1.8
pH 7.5
pH 9.0
FI
[SWNT](µM)
U-SWCNT
1G
2G
3G
B
Stern-Volmer
pH 1.8
pH 7.5
pH 9.0
I0/I
[SWNT](µM)
U-SWCNTs
1G
2G
3G

Fig. 5

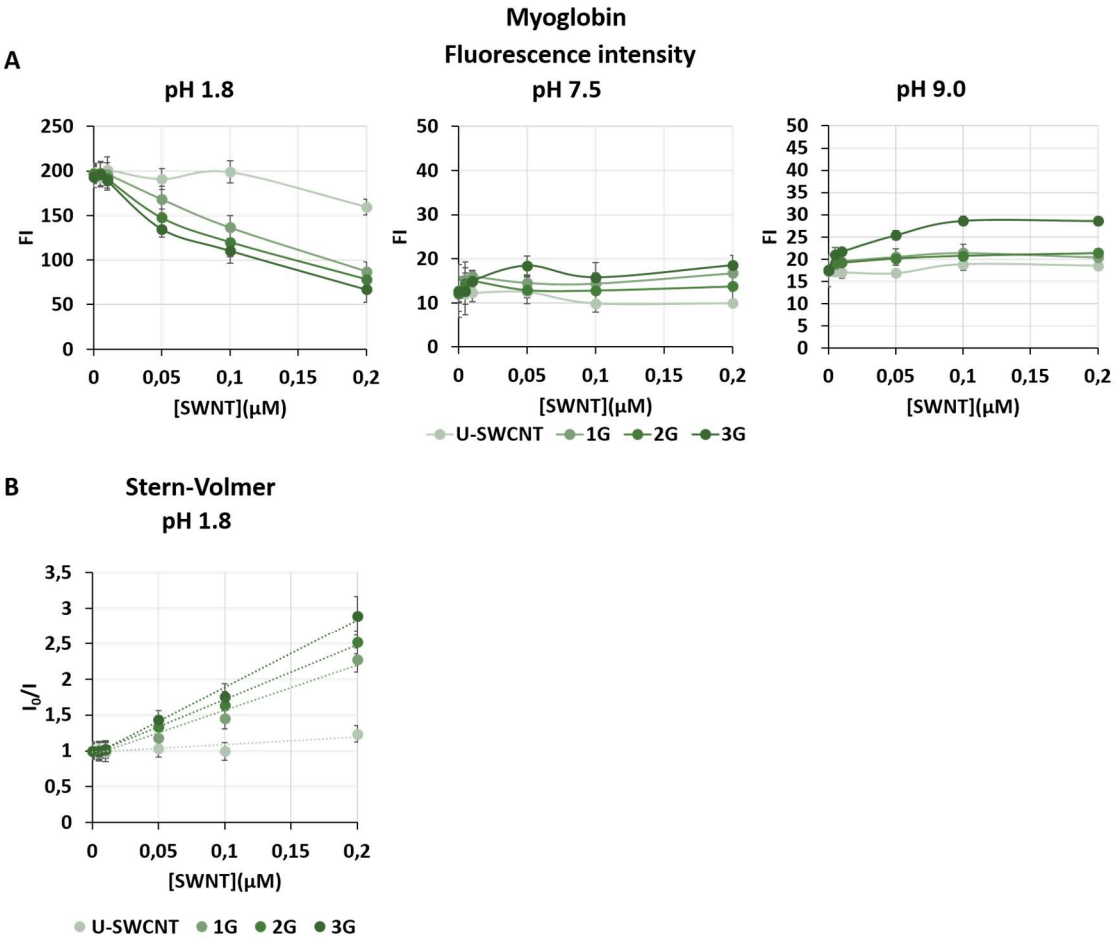
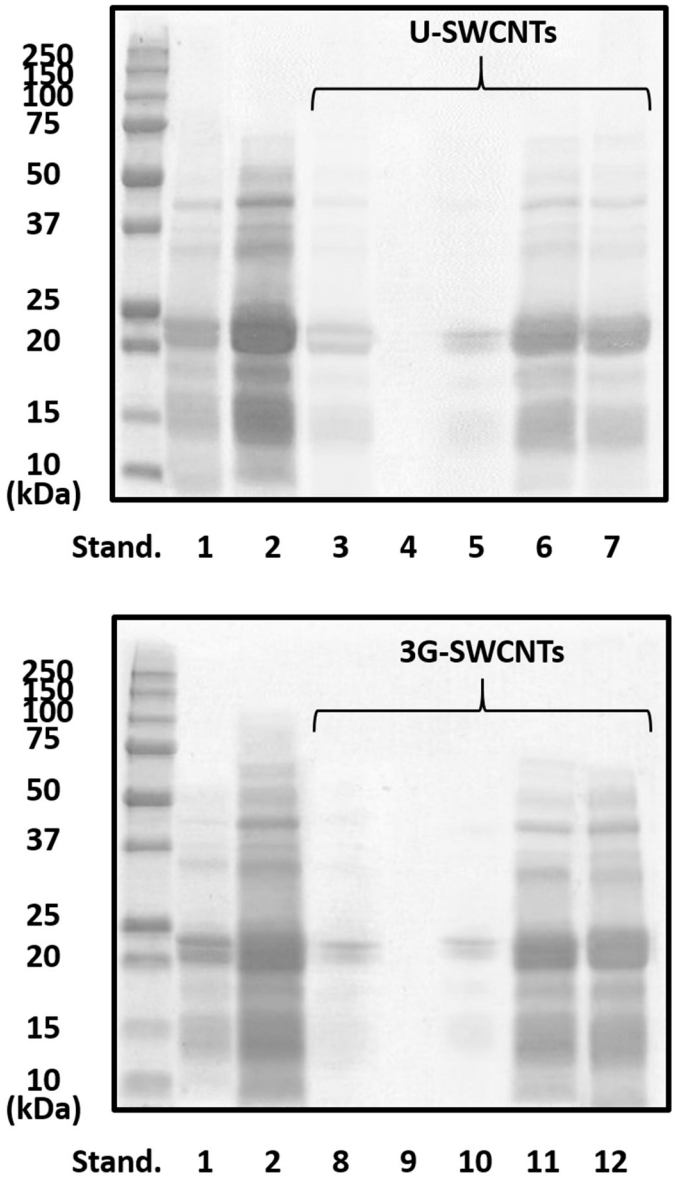

A
Myoglobin
Fluorescence intensity
pH 1.8
pH 7.5
pH 9.0
FI
[SWNT](μM)
U-SWCNT
1G
2G
3G
B
Stern-Volmer
pH 1.8
I0/I
[SWNT](μM)
U-SWCNT
1G
2G
3G

Fig. 6



	Protein Yield
1.	32.7 ± 2.1 %
2.	100.0 ± 1.7 %
3.	22.3 ± 1.1 %
4.	-
5.	10.3 ± 0.7 %
6.	73.3 ± 1.2 %
7.	71.1 ± 1.6 %
8.	12.7 ± 1.5 %
9.	-
10.	11.7 ± 1.3 %
11.	87.0 ± 1.7 %
12.	85.3 ± 0.9 %

Fig. 7