Surface biofunctionalization with liquid-phase plasma treatment of collagen molecules

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Article

Keywords: substrate biofunctionalization, collagen, liquid-phase plasma discharge

Posted Date: July 29th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-677830/v1>

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Title: Surface biofunctionalization with liquid-phase plasma treatment of collagen molecules

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33 **Abstract**

34 Surface functionalization is a key process in rendering various materials biocompatible.
35 Whereas a number of techniques and technologies have been developed for the purpose of
36 biofunctionalization, plasma treatment enables highly efficient surface modification.
37 Extending plasma treatment to biomolecules in the liquid phase will control
38 biofunctionalization via a simple process. However, interactions between plasma discharge
39 and biomolecules or solvents are poorly understood, potentially leading to the technical
40 limitation as to the utility of plasma treatment. In this study, we developed a technology for
41 substrate biofunctionalization that does not require surface modification but involves direct
42 treatment of a collagen molecules with liquid-phase plasma discharge. Biofunctionalization
43 of collagen by plasma treatment comprises three processes that increase its reactivity with
44 hydrophobic substrates: (1) charge-dependent changes in surface and interfacial properties
45 of the collagen solution; (2) local conformational changes of the collagen molecules without
46 their global structural alterations; and (3) induction of a micelle-like association formed by
47 collagen molecules. We anticipate such plasma-induced functionalization of protein
48 molecules to provide a versatile technique in the applications of biomaterials, including
49 those related to pharmaceuticals and cosmetics.

50 **Main text:**

51 **Introduction**

52 Biofunctionalization is important for treating disease and improving prognosis by increase in
53 the biocompatibility of implantable medical devices. Chemical modification of biomolecules
54 [1, 2] and surface modification with nonthermal plasma [3] are often used for
55 biofunctionalization of medical devices. Surface modification with nonthermal plasma in
56 particular enhance cell adhesiveness and the hydrophilicity of the medical devices such as
57 catheters, stents, and artificial bones and organs, which are typically constructed of heat-
58 sensitive materials, by a simple process [4-6]. Although plasma surface modification seems
59 to offer unlimited applications, there are some drawbacks. For example, plasma
60 modification results in uneven treatment of objects with fine and complex shapes. Micro-
61 spotting of the plasma source to overcome this problem makes the process more
62 complicated. Plasma modification is also not applicable to objects covered with highly
63 reactive materials or solutions because the plasma will react with such materials and
64 solutions before the flow reaches the target surface. Taking advantage of these drawbacks,
65 we hypothesized that functionalization of biomolecules in solution using direct plasma
66 treatment could uniformly enhance the cell adhesiveness of targets with fine and complex
67 shapes. A detailed understanding of the mechanisms through which plasma interacts with
68 biomolecules and solvent molecules will be necessary to validate our hypothesis (*i.e.*,
69 establishment of a biofunctionalization technology is possible). The interaction between
70 plasma and solution molecules has been clarified in terms of the transfer of charged
71 particles into the solution [7-9], the generation of reactive chemical species and ions [8, 10],
72 and oxidation-reduction reactions [11, 12]. These effects of plasma discharge can be used
73 to synthesize nanoparticles composed of polymers and metals [13-16]. However, our
74 understanding of how plasma interacts with biomolecules in solution, including proteins,
75 remains limited.

76 In this study, we developed a technology for the biofunctionalization of target
77 substrates without surface modification via direct treatment of a solution of collagen (an
78 extracellular matrix [ECM] protein) with liquid-phase plasma discharge. We found that
79 plasma treatment induced charge-dependent changes in the surface and interfacial
80 properties of the collagen solution. We also found that plasma treatment altered the local
81 conformation of the collagen molecules without affecting their global structure, inducing

them to assume a micelle-like association. These plasma-induced changes play key roles in substrate biofunctionalization.

Results

Characteristics of liquid-phase surface discharge plasma.

We first examined the characteristics of the liquid-phase surface discharge plasma source we previously developed (**Fig. 1a-c**; patent no. JP6713795B). This plasma source enables plasma treatment of biomolecules in solution by realizing a surface discharge in the liquid phase, which has been considered impossible. In the absence of a solution, plasma emission was observed at the tip of the applied electrode on the inner surface of the glass tube (**Fig. 1b**), and its spectra exhibited peaks at 315.9, 337.1, 357.7, 375.5, and 380.5 nm (**Fig. 1d**). These emission peaks, which are commonly found in atmospheric pressure plasma discharges, were attributed to the second positive system of nitrogen (N₂-SPS) [17]. The emission characteristics of this plasma source exhibited a similar trend, with some attenuation observed when ultrapure water, acetic acid solution, or collagen solution was introduced into the glass tube electrode (**Fig. 1e and f**, and **Supplementary Fig. 1**). Various chemical reactions occurred at the gas-liquid interface during plasma discharge, which resulted in the formation of oxidative products in the solution [8]. Our plasma source also produced oxidative compounds such as hydrogen peroxide (**Fig. 1g**), nitrite ion (**Fig. 1h**), and nitrate ion (**Fig. 1i**) in each solution. The concentrations of the oxidative compounds that were produced were generally low, ranging as high as 1.5 mg/L, although the concentrations varied slightly depending on solution type. Plasma treatment did not change the pH of the acetic acid or collagen solutions, which were buffered by acetic acid, but the pH of ultrapure water decreased from 6.3 to 5.0 upon plasma treatment (**Fig. 1j**).

Biofunctionalization of hydrophobic substrates with plasma-treated collagen.

We then evaluated the biofunctionalization of silicone-coated hydrophobic cover glass with collagen solution treated using the characterized plasma source. Human umbilical vein endothelial cells (HUVECs) adhered and spread on the hydrophobic cover glass during a 24-h incubation (P-Coll; **Fig. 2a**). In comparison with untreated collagen solution, acetic acid solution, and ultrapure water, the plasma-treated collagen coating encouraged the

113 adhesion of significantly more HUVECs and the formation of an endothelial monolayer (**Fig.**
114 **2a and b**). The plasma-treated collagen coating also stabilized the attachment of vinculin (a
115 focal adhesion–associated adaptor protein [18]) to each cell (**Fig. 2c-e**). The
116 biofunctionalization-enhancing effects of plasma-treated collagen were independent of the
117 coating processing time (**Supplementary Fig. 2**). In the proliferation assay, HUVECs
118 exhibited stable growth and formed a confluent monolayer without intercellular gaps on the
119 hydrophobic cover glass coated with plasma-treated collagen (**Fig. 2f and g**). Vinculin
120 attachment differed significantly between the plasma-treated collagen coating and the other
121 conditions (**Fig. 2h-j**), possibly because HUVECs are known to produce ECM proteins,
122 including collagen [19, 20]. These data thus demonstrate that plasma-treated collagen
123 coating enhances the biofunctionalization of hydrophobic substrates.

124

125 **Plasma-induced enhancement of the quality of collagen coating is a key factor in** 126 **biofunctionalization.**

127 We then investigated the cause of biofunctionalization (*i.e.*, why HUVECs adhere to and
128 proliferate better on hydrophobic cover glass coated with plasma-treated collagen solution).
129 Plasma-treated collagen was distributed uniformly on the hydrophobic cover glass, whereas
130 untreated collagen was distributed locally, and voids were apparent (**Fig. 3a**). We
131 hypothesized that the quality of the collagen coating affects the adhesion and proliferation
132 of HUVECs on the cover glass. This hypothesis is supported by quantitative analysis of the
133 data, which revealed a high average fluorescence intensity (**Fig. 3b**) and low degree of
134 variation in the plasma-treated collagen solution in the coated state (**Fig. 3c**). Focusing on
135 the wettability of the plasma-treated collagen solution, we therefore examined the surface
136 and interfacial properties, which are related to the solution's surface tension and contact
137 angle, respectively. Plasma treatment induced a time-dependent decrease in the contact
138 angle of the collagen solution on the hydrophobic cover glass (**Fig. 3d and e**). In ultrapure
139 water or acetic acid solution, the contact angle transiently decreased (up to 90 s after drop
140 adhesion) and then recovered to near the initial value (**Supplementary Fig. 3**). Plasma
141 treatment also produced a sustained decrease in the surface tension of the collagen
142 solution (**Fig. 3f**) and a transient decrease in the surface tension of other solutions
143 (**Supplementary Fig. 4**). The effect of evaporation on the results of contact angle analyses
144 cannot be neglected due to the small volume of a droplet (1.5 μL) [21]. Indeed, a time
145 course analysis of droplet size showed a clear decrease in volume (**Supplementary Movie**

1). However, surface tension measured using the capillary rise method, which is not affected by evaporation, exhibited a similar time-dependent decrease in plasma-treated collagen solution (**Fig. 3f**). We thus conclude that plasma treatment induces time-dependent variations in the surface and interfacial properties of collagen solution (*i.e.*, an increase in the wettability of the collagen solution and consequent enhancement of coating quality).

Electric charge regulates the surface and interfacial properties of the solution.

To examine the cause of the observed plasma-induced changes in the surface and interfacial properties of collagen solution, we considered the possibility that the hydrophobic surface of the cover glass undergoes modification by chemical species in the solution [22-24]. However, we could not confirm changes in the contact angle of collagen solutions containing hydrogen peroxide, nitrite ion, or nitrate ion similar to those observed in plasma-treated collagen solution (**Fig. 4a and b** and **Supplementary Fig. 5**). Similarly, no reduction in surface tension was observed following addition of these chemical species to the collagen solution (**Fig. 4c** and **Supplementary Fig. 6**). Even in ultrapure water, chemical species had no effect on the surface and interfacial properties (**Supplementary Fig. 7**). Hence, chemical species at concentrations generated in solution by plasma treatment (**Fig. 1h-j**) do not markedly affect the surface and interfacial properties, nor do the chemical species modify the surface of the hydrophobic cover glass.

We then investigated the effect of electric charge on surface and interfacial properties. Several previous studies have examined the effects of electric fields on contact angle and surface tension [25-27]. Using a polystyrene dish covered with a grounded aluminum sheet, we released the electric charge supplied to the collagen solution by plasma treatment. Release of the electric charge from the collagen solution eliminated plasma-induced time-dependent decreases in the solution's contact angle (**Fig. 4d and e**) and surface tension (**Fig. 4f**).

Our results suggest that electric charge is the primary factor mediating plasma-induced changes in surface and interfacial solution properties, leading to an increase in the wettability of the collagen solution. This scenario is supported by findings demonstrating that plasma-treated collagen solution in which the electric charge was released lost its effectiveness in promoting cell adhesion (**Fig. 4g and h**) and proliferation (**Fig. 4l and m**)

when coated on the hydrophobic substrate. The loss of the coating effect of plasma-treated collagen also affected the attachment of vinculin to HUVECs in the cell adhesion assay (Fig. 4i-k). However, loss of the coating effect did not decrease endothelial attachment of vinculin in the cell proliferation assay (Fig. 4n-p), and one possible reason for this is self-production of ECM proteins by the HUVECs [19, 20].

Plasma changes the local conformation and association of collagen molecules, which play crucial roles in biofunctionalization.

Even the collagen solution in which the charge was released after plasma treatment enhanced the biofunctionalization of the hydrophobic substrate to some extent upon coating for an extended period (Fig. 5a-e). We therefore hypothesized that plasma treatment induces changes in collagen molecules themselves which contribute to biofunctionalization, in addition to the changes induced in the surface and interfacial properties of the solution. We confirmed that the collagen molecules were thermally denatured by plasma treatment. In electrophoresis, we observed clear bands similar to those of untreated collagen, as opposed to the unclear band pattern of gelatinized collagen that was boiled for 1 h (Fig. 5f). Hence, plasma treatment did not induce any noticeable change in the molecular weight of collagen or in the abundance ratios of its secondary structures. In addition, plasma treatment did not denature small structures such as epitopes involved in antigen-antibody reactions (Fig. 5g).

To further examine the effects of plasma treatment on collagen molecules, we analyzed their structure in more detail. Circular dichroism spectra of collagen were similar regardless of the treatment condition (Fig. 5h). We confirmed that there were no significant changes in the secondary structure of collagen molecules specifically caused by plasma treatment. Focusing on the primary structure of collagen molecules, we then found that plasma treatment reduced the total amount of hydroxyproline, one of the amino acids [28] of which collagen is composed (Fig. 5i). This decrease in hydroxyproline content was not attributed specifically to plasma treatment but rather to the chemical species produced in the solution (Fig. 5j). These results led to the conclusion that the slight structural changes in collagen molecules due to chemical species do not directly contribute to biofunctionalization of the hydrophobic substrate.

In contrast, plasma treatment induced a slight increase in the viscosity of the collagen solution (**Supplementary Fig. 8**). We thus investigated the association and bonding states of the collagen molecules to elucidate the core mechanism of plasma treatment. Raman spectra of collagen solution after plasma treatment exhibited peaks specific to collagen (**Fig. 6a** and **Supplementary Fig. 9**). Two of these peaks were not derived from the chemical species produced by plasma treatment, and they remained in spectra of collagen solution in which the charge was released after plasma treatment (**Fig. 6a** and **b** and **Supplementary Fig. 9**). These two peaks are related to the local conformation around hydroxyproline (876 cm^{-1}) and proline (1445 cm^{-1} ; this peak indicates the absence of hydrogen bonding in X-PRO carbonyl groups) [29-32]. Hydroxyproline and proline play crucial roles in determining collagen stability [33] and permit the sharp twisting of the collagen triple helix [34]. Plasma treatment, therefore, induces changes in the local conformation, including hydrogen bonding states, of these amino acid side chains, which loosens the collagen helix.

We also measured the particle size of collagen associations based on dynamic light-scattering analysis (**Fig. 6c** and **d**). A comparison of peak values of collagen associations revealed that plasma treatment caused an increase in particle size (**Fig. 6e**). This was also true for the charge-released collagen solution, and the effect was not associated with chemical species (**Fig. 6f**). These results suggest that collagen molecules form micelle-like associations upon plasma treatment. This hypothesis is supported by our findings demonstrating that plasma treatment increased the fluorescence intensity of 8-anilino-1-naphthalenesulfonic acid (ANS) [35] in the collagen solution (**Fig. 6g**), indicating that plasma treatment alters the positions of hydrophobic sites. This alteration was also observed when the charge in the solution was released (**Fig. 6h**) but was not observed in a solution with added chemical species (**Fig. 6i**).

Based on these results, we conclude that plasma-induced changes in the local conformation and association of collagen molecules also play key roles in the biofunctionalization of hydrophobic surfaces. These findings can be explained by the fact that the surface and interfacial properties of a collagen solution differ from those of ultrapure water or acetic acid after plasma treatment (**Fig. 3e** and **f** and **Supplementary Figs. 3** and **4**).

Discussion

242 In this study, we developed a technology for the biofunctionalization of hydrophobic
243 substrates using plasma-activated collagen. This biofunctionalization is driven by three
244 processes that increase the reactivity of collagen molecules with hydrophobic substrates
245 (**Supplementary Fig. 10**): (1) plasma charges the collagen solution and then causes
246 alterations in the surface and interfacial properties of the solution, leading to an increase in
247 wettability on the hydrophobic surface; (2) plasma treatment changes the local
248 conformation of collagen molecules, including hydrogen bonding states, but does not
249 induce any significant changes in their global structure; and (3) interaction of collagen
250 molecules with plasma leads to forming their micelle-like associations. By better
251 understanding the plasma-induced functionalization of other biomolecules as well, a variety
252 of applications for plasma treatment could emerge, such as in pharmaceuticals and
253 cosmetics, not to mention the biofunctionalization of target substrates.

254 Plasma-induced mechanochemical or electrochemical effects lead to the
255 polymerization of molecules [36-38] and the synthesis of nanoparticles of polymers or
256 metals [13-16]. Such plasma polymerization and synthesis procedures are usually carried
257 out in the gas phase or at the gas-liquid interface. Gas-liquid interfacial plasma generates
258 OH radicals and other reactive species in solution, which can affect the polymerization of
259 polymers in solution. Our plasma source was discharged primarily in the liquid phase, with
260 intermittent switching between the gas and liquid phases, and did not induce
261 polymerization, (*i.e.*, gelation) of the collagen in solution. There was no noticeable change
262 in the molecular weight of collagen as a result of the plasma-induced oxidation-reduction
263 reaction. Plasma-induced changes in the local conformation and association of the collagen
264 molecules, as demonstrated in the present study, differ from the commonly known changes
265 associated with plasma polymerization and synthesis.

266 The mechanisms elucidated in the present study are based on indirect
267 measurements and considerations, particularly in regard to the local conformation and
268 association states of collagen molecules. We have not been able to directly determine how
269 plasma treatment changes the local conformation of collagen molecules, loosens the
270 collagen helix, or induces the micelle-like association of collagen molecules. This limitation
271 is attributed to the time-dependent nature of plasma-induced changes in the collagen
272 solution and collagen molecules, which makes it technically difficult to perform assays that
273 are typically time consuming, such as structural analyses. The solution in-flow rate was
274 fixed at 1 mL/min in view of the temperature increase caused by plasma treatment. By

275 slowing the in-flow rate while controlling the temperature, it becomes possible to increase
276 the efficiency of collagen functionalization, as revealed in this study. We believe that
277 consideration of the treatment conditions will enable us to sustain the plasma-induced
278 functionalization of collagen and fully elucidate the underlying mechanisms.

279 **Methods**

280 **Collagen solution.** Collagen, a common ECM protein, was used for target
281 biofunctionalization. A solution of collagen I, rat tail (A10483-01, Gibco, Thermo Fisher
282 Scientific, Waltham, MA, USA and 08-115, Merck, Darmstadt, Germany) was prepared at
283 50–1000 µg/mL by dissolving in 20 mM acetic acid (017-00256, Wako Pure Chemical
284 Industries, Osaka Japan). For comparison, we also treated ultrapure water and 20 mM
285 acetic acid with surface discharge plasma.

286 **Surface discharge plasma and its characterization.** Surface discharge plasma was
287 generated in a borosilicate glass tube with outer and inner diameters of 8 mm and 6 mm,
288 respectively (**Fig. 1a** and **b**). The pulse voltage was applied to an aluminum sheet electrode
289 attached onto the inner surface of the tube. An aluminum electrode attached on the outside
290 across the glass was grounded. The applied voltage was +8 kV from 0 to peak, with a
291 frequency of 5 kHz (**Fig. 1c**), which was confirmed using a high-voltage probe (P6015A,
292 Tektronix, Beaverton, OR, USA).

293 Light emission from the charged plasma was recorded with a digital camera (X-T4,
294 Fujifilm, Tokyo, Japan) equipped with a macro lens (XF80mmF2.8 R LM OIS WR Macro,
295 Fujifilm). Emission spectra were measured using a high-resolution spectrometer
296 (HR4000CG-UV-NIR, Ocean Insight, Orlando, FL, USA).

297 **Surface discharge plasma treatment.** The prepared solution was in-flowed through a
298 fluorocarbon polymer tube into the glass tube electrode using a syringe pump (Legato 110,
299 KD Scientific, Holliston, MA, USA). The in-flowed solution was then treated with surface
300 discharge plasma. The in-flow rate of the solution was 1 mL/min. Treated solution was
301 collected in a polystyrene dish (3000-035, AGC Techno Glass, Shizuoka, Japan). To
302 exclude any effects of electric charge, we also collected the solution in a polystyrene dish
303 covered with a grounded aluminum sheet.

304 **Chemical species detection.** The production of hydrogen peroxide, nitrite ion, and nitrate
305 ion was examined because these species are reportedly generated by plasma-liquid
306 interactions [8]. Hydrogen peroxide, nitrite ions, and nitrate ions present in the solutions
307 (ultrapure water, 20 mM acetic acid solution, and 50 µg/mL collagen solution) were
308 detected using 4-aminoantipyrine colorimetric assay (WAK-H₂O₂, Kyoritsu Chemical-
309 Check Lab, Tokyo, Japan), naphthyl ethylenediamine colorimetric assay (WAK-NO₂, WAK-
310 NO₃, Kyoritsu Chemical-Check Lab), and Griess assay (NK05, Dojindo Laboratories,

311 Kumamoto, Japan) kits, respectively. The absorbance was monitored in each assay using
312 an absorptiometer (DPM-MTSP, Kyoritsu Chemical-Check Lab or iMark, Bio-Rad
313 Laboratories, Hercules, CA, USA). The concentration of each chemical species was then
314 determined based on the measured absorbance and a calibration curve. The pH of each
315 solution was measured using a pH meter (LAQUAtwin; AS-pH-11B, AS ONE, Osaka,
316 Japan).

317 **Substrate preparation.** A silicone-coated hydrophobic cover glass (C2240, Matsunami
318 Glass Industry, Osaka, Japan) was biofunctionalized with plasma-treated collagen solution.
319 The hydrophobic cover glass was immersed in the plasma-treated collagen solution (50
320 µg/mL) for 3 min and then allowed to completely air dry. For comparison, a cover glass was
321 also immersed in untreated collagen solution, 20 mM acetic acid, or ultrapure water for 3
322 min. To evaluate the effect of treatment time on biofunctionalization, we also examined
323 hydrophobic cover glass samples immersed in plasma-treated or untreated collagen
324 solution for 1 h.

325 **Antibodies.** Mouse monoclonal anti-vinculin antibody (26520-1-AP, dilution; 1:500
326 [Immuno-fluorescence; IF]) was purchased from Proteintech (Rosemont, IL, USA). Mouse
327 monoclonal anti-VE-cadherin antibody (sc-9989, dilution; 1:100 [IF]) and mouse monoclonal
328 anti-COL1A antibody (sc-59772, dilution; 1:100 [IF] and 1:1000 [Western blotting; WB])
329 were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Alexa Fluor 488–
330 conjugated goat anti-mouse IgG (A28175, dilution; 1:200 [IF]), Alexa Fluor 594–conjugated
331 goat anti-mouse IgG (A11032, dilution; 1:200 [IF]), and Alexa Fluor 488–conjugated goat
332 anti-rabbit IgG (A27034, dilution; 1:200 [IF]) secondary antibodies were purchased from
333 Thermo Fisher Scientific. Horseradish peroxidase–conjugated anti-mouse IgG (7076S,
334 dilution; 1:5000 [WB]) was purchased from Cell Signaling Technology (Danvers, MA, USA).

335 **Cell culture.** HUVECs (200-05n, Cell Applications, San Diego, CA, USA) were cultured in
336 Medium 199 (31100-035, Gibco, Thermo Fisher Scientific) containing 20% heat-inactivated
337 fetal bovine serum (12483-020, Gibco), 10 µg/L human basic fibroblast growth factor (GF-
338 030-3, Austral Biologicals, San Ramon, CA, USA), and 1% penicillin/streptomycin (15140-
339 122, Gibco). HUVECs from the fifth to ninth passages were used for experiments in this
340 study.

341 **Cell adhesion assay.** HUVECs were seeded on the prepared glass substrate at a density
342 of 4×10^4 cells/cm². The cells were then incubated with culture medium in a CO₂ incubator.

343 After 24 h of incubation, adherent HUVECs were observed using a phase-contrast
344 microscope (CKX41, Olympus, Tokyo, Japan) equipped with a sCMOS camera
345 (WRAYCAM-SR 130 M, WRAYMER, Osaka, Japan). Based on the captured images, the
346 number of cells per unit area was determined using ImageJ Fiji software (US National
347 Institutes of Health) [39].

348 **Cell proliferation assay.** HUVECs were seeded on the prepared glass substrate at a
349 density of 1×10^4 cells/cm². The cells were then incubated with culture medium in a CO₂
350 incubator for up to 72 h, with replacement of the medium every day. Images of HUVEC
351 proliferation were captured using a phase-contrast microscope every 24 h after cell
352 seeding. The number of cells per unit area was then determined based on the captured
353 images.

354 **Immunofluorescence staining.** After cell adhesion and proliferation assays, HUVECs
355 were fixed with 4% paraformaldehyde phosphate buffer solution (163-20145, Fujifilm Wako
356 Pure Chemical Corporation, Osaka, Japan). The cells were permeabilized with 0.1%
357 TritonX-100 (17-1315-01, Pharmacia Biotech, Uppsala, Sweden) in phosphate-buffered
358 saline (PBS; 05913, Nissui Pharmaceutical, Tokyo, Japan) and incubated in 1% Block Ace
359 (BA; UKB40, DS Pharma Biomedical, Osaka, Japan) to prevent nonspecific antibody
360 adsorption. The cells were then stained using primary and secondary antibodies diluted in
361 1% BA in PBS and PBS, respectively, at predefined concentrations. Cell nuclei and actin
362 filaments were stained using 4',6-diamidino-2-phenylindole (D1306, Thermo Fisher
363 Scientific) and Alexa Fluor 488–conjugated phalloidin (A12379, Thermo Fisher Scientific),
364 respectively. Stained HUVECs were observed using a wide-field fluorescence microscope
365 (BX51WI, Olympus). For staining of collagen coated onto the hydrophobic cover glass, the
366 blocking process and subsequent steps described above were performed.

367 **Vinculin attachment points.** Adhesion of cells to the biofunctionalized glass substrate was
368 evaluated based on the number of attachment points of a focal adhesion–associated
369 adaptor protein, vinculin. We first prepared a fluorescence image minus background noise
370 based on the fluorescence intensity threshold using the function “subtract background-
371 rolling” in ImageJ Fiji. The number of vinculin particles was then counted as vinculin
372 attachment points from the binarized images using the function “analyze particles”. The
373 number of cells was determined based on counting of fluorescent-stained cell nuclei and
374 calculated vinculin attachment points per cell.

375 **Fluorescence intensity of COL1A.** Fluorescence images of six arbitrary locations were
376 captured for each hydrophobic cover glass. Fluorescence intensity and standard deviation
377 of stained COL1A were determined for a rectangular region of 3,000 × 3,000 pixels (240 ×
378 240 μm) using ImageJ Fiji.

379 **Contact angle analysis.** To evaluate the wettability of the plasma-treated solutions
380 (ultrapure water, 20 mM acetic acid solution, and 50 μg/mL collagen solution), the contact
381 angle was measured on the silicone-coated hydrophobic cover glasses using a contact
382 angle meter (Smart Contact Mobile 4, Excimer Inc., Yokohama, Japan). Images of 1.5-μL
383 solution droplets were captured every 10 s from 150 s to 300 s after the start of surface
384 discharge plasma treatment. The contact angle was calculated based on the θ -2 θ method
385 using ImageJ Fiji.

386 **Surface tension measurement.** Surface tension of the solution was measured based on
387 the capillary rise method [40] using a borosilicate glass capillary (9600150, Ringcaps®
388 Disposable Capillary Pipettes 1 Mark 50 μL, Hirschmann Laborgeräte GmbH & Co.,
389 Eberstadt, Germany) with inner diameter of 1.05 mm. Capillary action (*i.e.* rise of the
390 solution [ultrapure water, 20 mM acetic acid solution, or 50 μg/mL collagen solution]) at
391 150, 240, and 300 s after the start of plasma treatment was photographed using a digital
392 camera and macro lens. The contact angle of the solution at the capillary inner wall and the
393 height of the solution level were measured using ImageJ Fiji. The surface tension, γ , was
394 obtained from the following equation:

395
$$\gamma = r\rho g / 2\cos\theta,$$

396 where r , ρ , g , and θ represent the inner radius of the glass capillary, the solution density,
397 the gravitational acceleration, and the contact angle of the solution, respectively.

398 **Electrophoresis and immunoblotting.** Collagen molecules were collected from the
399 plasma-treated solution (50 μg/mL collagen solution) according to the following protocol.
400 Acetic acid buffer (20 mM) was removed from the plasma-treated collagen solutions (480
401 μL each) after washing twice with ultrapure water and centrifugation (20,000g at 4°C for 10
402 min) using an ultrafiltration spin column (PT-1002, Integrale, Tokyo, Japan). The extracted
403 collagen molecules were resuspended in Laemmli sample buffer (161-0747, Bio-Rad
404 Laboratories) containing 20 mM dithiothreitol (161-0611, Bio-Rad Laboratories). Samples
405 (12 μg collagen each) were subjected to SDS-PAGE using a Criterion TGX gel (gel
406 concentration: 10%; 567-1034J10, Bio-Rad Laboratories). The gel was stained with

407 Coomassie Brilliant Blue (161-0436, Bio-Rad Laboratories) and then scanned with
408 ChemiDoc XRS+ (Bio-Rad Laboratories).

409 After SDS-PAGE, samples were also transferred onto an Immun-Blot PVDF
410 membrane (162-0177, Bio-Rad Laboratories). The membrane was blocked with Tris-
411 buffered saline containing 1% BA and 0.05% Tween 20 and then stained using primary and
412 secondary antibodies diluted in Can Get Signal Immunoreaction Enhancer Solution (NKB-
413 101, Toyobo, Osaka, Japan). The blotted collagen bands were detected and visualized
414 using Clarity Western ECL Substrate (170-5061, Bio-Rad Laboratories). The molecular
415 weight of each sample was determined based on Precision Plus Protein Dual Color
416 Standards (161-0374, Bio-Rad Laboratories).

417 Collagen solutions not treated with plasma and collagen solution boiled for 1 h were
418 also analyzed for comparison.

419 **Viscosity measurement.** Viscosity of the plasma-treated collagen solution (50 µg/mL) was
420 analyzed using a viscometer (VM-10A, SEKONIC, Tokyo, Japan). The volume of solution
421 required for the measurement was approximately 5 mL. The temperature change of the
422 solution during measurement was adjusted to $\pm 0.5^{\circ}\text{C}$. The temperature of the solution was
423 measured using a digital thermometer (CT-320WP, Custom, Tokyo, Japan).

424 **Circular dichroism spectroscopy.** Circular dichroism spectra were collected to visualize
425 the secondary structure of collagen molecules. Plasma-treated collagen solution (50 µg/mL)
426 was placed into a quartz glass cell (S15-UV-2, GL Sciences, Tokyo, Japan), and the
427 spectra were collected using a circular dichroism spectrometer (J-1100, JASCO
428 Corporation, Tokyo, Japan). Spectra of the solvent (20 mM acetic acid solution) were
429 subtracted from the results as background.

430 **Hydroxyproline assay.** Samples were prepared by acid hydrolysis of collagen by adding
431 hydrochloric acid to plasma-treated collagen solution (1 mg/mL) to a final concentration of 4
432 M, followed by boiling for 1 h. The amount of hydroxyproline inside the collagen molecules
433 was determined using a hydroxyproline assay kit (QZBhypro1, QuickZyme Biosciences,
434 Leiden, Netherlands) according to the manufacturer's instructions.

435 **Raman spectroscopy.** Raman spectra of plasma-treated collagen solution (50 µg/mL)
436 were collected using a spontaneous Raman spectroscopy system consisting of an EM-CCD
437 camera, a single-mode laser (wavelength = 532 nm), and spectrometer (center wavelength

438 = 570 nm) [41, 42]. After plasma treatment, the solution was placed in a quartz glass
439 cuvette (1 × 10 mm in dimensions) for analysis (S15-UV-1, GL Sciences, Tokyo, Japan),
440 and Raman spectra were obtained at the center of the optical path. Each exposure time
441 was set to 3 min, and the spectra were calculated from the accumulation of 10
442 measurements.

443 **Particle size analysis.** The size distribution of collagen associations was measured based
444 on dynamic light scattering using a Zetasizer Nano (ZSP, Malvern Panalytical Ltd., Malvern,
445 UK). Two milliliters of plasma-treated collagen solution was transferred to a polystyrene
446 cuvette (67.754, Sarstedt, Nümbrecht, Germany) for measurement immediately after
447 treatment, and the measurement chamber was kept at 25°C. Each exposure time was set
448 to 10 s, and the size distribution was calculated from 11 accumulated measurements. Four
449 measurements were obtained for each of three samples. Peak values were evaluated by
450 excluding particles >100 nm in size (**Fig. 6c** and **e**), which were considered debris
451 originating from electrode erosion caused by the plasma discharge [43, 44]. In the
452 experiment examining the effect of chemical species, no debris was observed because the
453 samples were prepared without using the discharge apparatus (**Fig. 6d** and **f**).

454 **ANS binding assay.** Conformational changes in collagen molecules were evaluated based
455 on the fluorescence intensity of ANS (A0472, Tokyo Chemical Industry Corporation, Tokyo,
456 Japan). The fluorescent properties of ANS change as the molecule reacts with hydrophobic
457 regions on the protein surface [35]. Comparisons of fluorescence intensities can thus give
458 information about the transition between the hydrophilic and hydrophobic states of collagen
459 molecules triggered by plasma treatment. ANS was added to 50 µg/mL collagen solution to
460 achieve a final concentration of 1 mM. The fluorescence intensity of ANS in each solution
461 was measured with excitation at 370 nm and emission at 400–600 nm using a multi-grating
462 microplate reader (SH-9000Lab, Corona Electric Corporation, Ibaraki, Japan).

463 **Statistics and reproducibility.** All values are shown as mean ± standard deviation (SD)
464 unless stated otherwise. All data were obtained from at least three independently repeated
465 experiments. Statistical significance was evaluated using the two-sided Welch's *t* test with
466 Bonferroni correction for multiple comparisons, with statistical significance set at $p \leq 0.05$.

467 **Data availability**

468 The authors declare that all data supporting the findings of this study are available within
469 this article and its supplementary information files or from the corresponding author upon
470 reasonable request.

471 **Conflicts of interest**

472 Patents associated with the surface discharge plasma source and technique for
473 biofunctionalization using the source have been registered in Japan (JP6408689B,
474 JP6713795B).

475 **Acknowledgements**

476 The authors thank Prof. M. Ukai of Tokyo University of Agriculture and Technology for his
477 expert advice on plasma emission spectroscopy. The Japan Society for the Promotion of
478 Science (JSPS KAKENHI grant number 18K19894) and Japan Science and Technology
479 Agency (Building of Consortia for the Development of Human Resources in Science and
480 Technology) are acknowledged for financial support.

481 **Author contributions**

482 D.Y. conceived and designed the research. S.T. conducted most of the experiments. S.K.
483 and M.O. conducted ANS binding assays. K.I. and K.M. were responsible for Raman
484 spectroscopy. K.F. conducted viscosity measurements. G.M. provided support for plasma
485 spectrometric analyses. T.M. provided technical assistance for circular dichroism
486 spectroscopy and particle size analyses. All authors discussed the data. S.T. and D.Y.
487 wrote the manuscript. D.Y. directed and supervised the project.

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Fig. 1. Surface discharge plasma source. (a) Electrode design and the apparatus for plasma treatment of collagen solution. (b) Cross section of the electrode and light emitted by surface discharge plasma. (c) Waveform of the applied voltage. (d) Emission spectrum of plasma discharge in the gas phase. Typical peaks are attributed to the N₂-SPS(C-B). (e) Images of light emitted by the plasma generated in each solution. (f) Emission spectrum of

619 plasma discharge in the collagen solution. As in the case of discharge in the gas phase, a
620 peak typical of the N₂-SPS(C–B) was observed. Concentrations of dissolved **(g)** H₂O₂
621 (mean + SD, *n* = 20 experiments), **(h)** NO₂[–] (mean + SD, *n* = 20 experiments), and **(i)** NO₃[–]
622 (mean + SD, *n* = 5 experiments) in each solution after plasma treatment. **(j)** Change in pH
623 of each solution before and after plasma treatment (mean + SD, *n* = 3 experiments). UPW,
624 ultrapure water; AA, 20 mM acetic acid; Coll, 50 µg/mL collagen solution.

Fig. 2. Biofunctionalization of silicone-coated hydrophobic cover glass with plasma-treated collagen solution. (a) Adhesion of endothelial cells on hydrophobic cover glass pre-coated with each solution. Scale bars, 200 μm . (b) Number of adhered HUVECs in a 1-mm² area 24 h after seeding. Whiskers represent the 10th and 90th percentiles, the box represents the 25th to 75th percentiles, the central line depicts the median, and the white square inside each box indicates the average value. Each value was obtained from 15 images, which were captured from three independently repeated experiments ($n = 15$ images). (c) Representative fluorescence images of VE-cadherin and vinculin in adherent cells 24 h after seeding. Scale bars, 50 μm . (d) Quantification of vinculin attachment points in an adherent cell calculated based on (e) the number of cells in each image. Each value is shown as a box-and-whisker plot obtained from 20 images in three independently repeated experiments ($n = 20$ images). (f) Proliferation of HUVECs on hydrophobic cover glass pre-coated with each solution. Scale bars, 200 μm . (g) Number of HUVECs in a 1-mm² area 24 to 72 h after seeding. Each value is shown as a box-and-whisker plot obtained from 15 images in three independently repeated experiments ($n = 15$ images). (h) Representative fluorescence images of VE-cadherin and vinculin in cells proliferating on hydrophobic cover glass 72 h after seeding. Scale bars, 50 μm . (i) Quantification of vinculin attachment points in a proliferated cell calculated based on (j) the number of cells in each image. Each value is shown as a box-and-whisker plot obtained from 20 images in three independently repeated experiments ($n = 20$ images). NT, non-treated; AA, 20 mM acetic acid; Coll, 50 $\mu\text{g/mL}$ collagen solution; P-Coll, plasma-treated collagen solution.

Fig. 3. Collagen coating quality derives from plasma-induced changes in surface and interfacial properties and contributes to substrate biofunctionalization. (a) Representative fluorescence images of collagen (COL1A) on hydrophobic cover glass. Scale bars, 5 mm (left), 50 μm (middle), and 10 μm (right). **(b, c)** Quantification of coating quality. **(b)** Averaged fluorescence intensity and **(c)** standard deviation of fluorescence intensity of COL1A in a rectangular region ($240 \times 240 \mu\text{m}$). Each value is shown as a box-and-whisker plot obtained from 30 images in six independently repeated experiments ($n = 30$ images). **(d)** Time sequence images of a collagen solution droplet on hydrophobic cover glass. **(e)** Temporal change in contact angle of the collagen solution droplet (mean $\pm$ SD, $n = 20$ experiments). **(f)** Variation over time of surface tension of the collagen solution. Each value is shown as a box-and-whisker plot obtained from 20 independently repeated experiments ($n = 20$ experiments). Coll, 50 $\mu\text{g/mL}$ collagen solution; P-Coll, plasma-treated collagen solution.

Fig. 4. Electric charge regulates the surface and interfacial properties of the solution, leading to substrate biofunctionalization. (a) Time sequence images of a droplet of collagen solution containing chemical species on hydrophobic cover glass. (b) Contact angle of a droplet of collagen solution containing chemical species (mean $\pm$ SD, $n = 5$ experiments). (c) Temporal variation of the surface tension of collagen solution containing chemical species. Each value is shown as a box-and-whisker plot obtained from five independently repeated experiments ($n = 5$ experiments). (d) Time sequence images of a collagen solution droplet on hydrophobic cover glass. (e) Contact angle of a droplet of collagen solution in which the charge was released after plasma treatment (mean $\pm$ SD, $n = 5$ experiments). (f) Temporal variation of the surface tension of collagen solution in which the charge was released after plasma treatment. Each value is shown as a box-and-whisker plot obtained from five independently repeated experiments ($n = 5$ experiments). (g) Phase-contrast images of HUVECs adhered onto hydrophobic cover glass pre-coated with each solution. Scale bars, 200 μm . (h) Number of adherent HUVECs in a 1-mm² area 24 h after seeding. Each value is shown as a box-and-whisker plot obtained from 15 images in three independently repeated experiments ($n = 15$ images). (i) Representative fluorescence images of VE-cadherin and vinculin in adherent cells 24 h after seeding. Scale bars, 50 μm . (j) Quantification of vinculin attachment points in an adherent cell calculated based on (k) the number of cells in each image. Each value is shown as a box-and-whisker plot obtained from 20 images in three independently repeated experiments ($n = 20$ images). (l) Phase-contrast images of cell proliferation on hydrophobic cover glass pre-coated with each solution. Scale bars, 200 μm . (m) Number of HUVECs in a 1-mm² area 24 to 72 h after seeding. Each value is shown as a box-and-whisker plot obtained from 15 images in three independently repeated experiments ($n = 15$ images). (n) Representative fluorescence images of VE-cadherin and vinculin in cells proliferating on hydrophobic cover glass 72 h after seeding. Scale bars, 50 μm . (o) Quantification of vinculin attachment points in a proliferated cell calculated based on (p) the number of cells in each image. Each value is shown as a box-and-whisker plot obtained from 20 images in three independently repeated experiments ($n = 20$ images). Coll, 50 $\mu\text{g/mL}$ collagen solution; Coll + Chem, collagen solution containing H₂O₂, NO₂⁻, and NO₃⁻ at concentrations equal to those produced by plasma treatment; P-Coll, plasma-treated collagen solution; G-Coll, collagen solution in which the charge was released after plasma treatment.

Fig. 5. Coating for an extended period enhances biofunctionalization regardless of the charge state of the collagen solution, but plasma treatment does not cause thermal denaturation or significant structural changes in collagen. (a) HUVECs adhering onto hydrophobic cover glass pre-coated with each solution for 1 h. Scale bars, 200 μ m. **(b)** Number of adherent HUVECs in a 1-mm² area 24 h after seeding. Each value is shown as a box-and-whisker plot obtained from 15 images in three independently repeated experiments ($n = 15$ images). **(c)** Representative fluorescence images of VE-

702 cadherin and vinculin in HUVECs 24 h after seeding. Scale bars, 50 μ m. **(d)** Quantification
703 of vinculin attachment points in a cell calculated based on **(e)** the number of cells in each
704 image. Each value is shown as a box-and-whisker plot obtained from 20 images in three
705 independently repeated experiments ($n = 20$ images). **(f)** Representative CBB-stained
706 electrophoresis gel of collagen molecules in solution and **(g)** representative blot of COL1A.
707 Images are representative of three independent experiments with similar results ($n = 3$
708 experiments). **(h)** Averaged circular dichroism spectra of collagen solution ($n = 5$
709 experiments). **(i, j)** Amount of hydroxyproline in each collagen solution ($n = 3$ experiments).
710 Coll, 50 μ g/mL collagen solution; P-Coll, plasma-treated collagen solution; G-Coll, collagen
711 solution in which the charge was released after plasma treatment; B-Coll, collagen solution
712 boiled for 1 h; Coll + Chem, collagen solution containing H_2O_2 , NO_2^- , and NO_3^- at
713 concentrations equal to those produced by plasma treatment.

Fig. 6. Plasma induces changes in the local conformation and association state of collagen molecules, leading to biofunctionalization of hydrophobic substrates. (a, b) Difference in Raman spectra intensity between each solution and 20 mM acetic acid. Data

718 shown are representative of three independent experiments with similar results ($n = 3$
719 experiments). HYP, hydroxyproline; PRO, proline. **(c, d)** Size distribution of collagen
720 associations in each solution. **(e, f)** Peak size of collagen associations extracted from 12
721 measurement results based on three independent samples ($n = 12$ measurement results).
722 Peak values were evaluated excluding particles >100 nm in size, which represent plasma
723 discharge–related debris originating from the electrodes. **(g, h, i)** Averaged ANS-
724 fluorescence intensity in collagen solution ($n = 6$ measurement results; **g, h**, and $n = 7$
725 measurement results, **i**). Coll, 50 $\mu\text{g/mL}$ collagen solution; P-Coll, plasma-treated collagen
726 solution; G-Coll, collagen solution in which the charge was released after plasma treatment.
727 Coll + Chem, collagen solution containing H_2O_2 , NO_2^- , and NO_3^- at concentrations equal to
728 those produced by plasma treatment.

Supplementary Fig. 1. Emission spectra of plasma discharge in each solution. (a) Ultrapure water (UPW) and **(b)** 20 mM acetic acid (AA). The typical peaks are attributed to the N₂-SPS(C-B).

Supplementary Fig. 2. Effect of coating with plasma-treated collagen solution for an extended period of time on biofunctionalization of a hydrophobic cover glass. (a) Adhesion of endothelial cells onto hydrophobic cover glass pre-coated with collagen solution for 1 h. Scale bars, 200 μ m. **(b)** Number of adhered HUVECs in a 1-mm² area 24 h after seeding. Whiskers represent the 10th and 90th percentiles, the box represents the 25th to 75th percentiles, the central line depicts the median, and the white square inside each box indicates the average value. Each value was obtained from 15 images captured from three independently repeated experiments ($n = 15$ images). **(c)** Representative fluorescence images of VE-cadherin and vinculin in adherent cells 24 h after seeding. Scale bars, 50 μ m. **(d)** Quantification of vinculin attachment points in an adherent cell calculated based on **(e)** the number of cells in each image. Each value is shown as a box-and-whisker plot obtained from 20 images in three independently repeated experiments ($n = 20$ images). Coll, 50 μ g/mL collagen solution; P-Coll, plasma-treated collagen solution.

Supplementary Fig. 3. Plasma induces transient changes in interfacial properties between the hydrophobic substrate and the solution. (a) Time sequence images of an ultrapure water (UPW) droplet on hydrophobic cover glass. (b) Temporal variation of the contact angle of an UPW droplet (mean $\pm$ SD, $n = 20$ experiments). (c) Time sequence images of a 20 mM acetic acid (AA) droplet on hydrophobic cover glass. (d) Temporal variation of the contact angle of an AA droplet (mean $\pm$ SD, $n = 20$ experiments).

Supplementary Fig. 4. Plasma induces transient changes in surface properties of the solution. Temporal variation of the surface tension of **(a)** ultrapure water (UPW) and **(b)** 20 mM acetic acid (AA). Whiskers represent the 10th and 90th percentiles, the box represents the 25th to 75th percentiles, the central line depicts the median, and the white square inside each box indicates the average value. Each value was obtained from 20 independently repeated experiments ($n = 20$ experiments).

Supplementary Fig. 5. Chemical species cause no change in interfacial properties between the hydrophobic substrate and collagen solution. Time sequence images of a droplet of 50 $\mu\text{g/mL}$ collagen solution containing (a) H_2O_2 , (c) NO_2^- , or (e) NO_3^- on hydrophobic cover glass. Contact angle of a droplet of 50 $\mu\text{g/mL}$ collagen solution containing (b) H_2O_2 , (d) NO_2^- , or (f) NO_3^- (mean $\pm$ SD, $n = 5$ experiments).

Supplementary Fig. 6. Chemical species do not change the surface properties of collagen solution resulting from plasma treatment. Surface tension of 50 $\mu\text{g/mL}$ collagen solution containing chemical species. Whiskers represent the 10th and 90th percentiles, the box represents the 25th to 75th percentiles, the central line depicts the median, and the white square inside each box indicates the average value. Each value was obtained from five independently repeated experiments ($n = 5$ experiments). Coll + Chem, collagen solution containing H₂O₂, NO₂⁻, and NO₃⁻ at concentrations equal to those produced by plasma treatment.

777 **Supplementary Fig. 7. Chemical species cause no changes in surface and interfacial**
778 **properties of ultrapure water (UPW).** Time sequence images of a droplet of UPW
779 containing **(a)** H_2O_2 , **(c)** NO_2^- , or **(e)** NO_3^- on hydrophobic cover glass. Contact angle of a
780 droplet of UPW containing **(b)** H_2O_2 , **(d)** NO_2^- , or **(f)** NO_3^- (mean $\pm$ SD, $n = 5$ experiments).
781 **(i)** Surface tension of UPW containing chemical species. Whiskers represent the 10th and
782 90th percentiles, the box represents the 25th to 75th percentiles, the central line depicts the
783 median, and the white square inside each box indicates the average value. Each value was
784 obtained from five independently repeated experiments ($n = 5$ experiments). UPW + Chem,
785 UPW containing H_2O_2 , NO_2^- , and NO_3^- at concentrations equal to those produced by
786 plasma treatment.

Supplementary Fig. 8. Plasma treatment induces a slight increase in the viscosity of collagen solution. (a) Viscosity of collagen solution before and after plasma treatment. **(b)** Difference in the viscosity of collagen solution before and after plasma treatment ($n = 10$ experiments). **(c)** Viscosity of collagen solution under various conditions. **(d)** Difference in the viscosity of collagen solution before and after plasma treatment ($n = 10$ experiments). Slight increase in viscosity was also observed in collagen solution in which the charge was released after plasma treatment. Coll, 50 $\mu\text{g/mL}$ collagen solution; P-Coll, plasma-treated collagen solution; G-Coll, collagen solution in which the charge was released after plasma treatment.

Supplementary Fig. 9. Raman spectral waveform of collagen solution under various conditions. (a) 20 mM acetic acid solution (AA) as base. (b) 50 µg/mL collagen solution (Coll). (c) Collagen solution after plasma treatment (P-Coll). (d) Collagen solution in which the charge was released after plasma treatment (G-Coll). (e) 20 mM AA solution as base. (f) 50 µg/mL Coll. (g) Collagen solution after plasma treatment (P-Coll). (h) Collagen solution containing H₂O₂, NO₂⁻, and NO₃⁻ at concentrations equal to those produced by plasma treatment (Coll + Chem). These Raman spectral waveforms were used to calculate the difference in Raman intensities (Fig. 6a and b) Fig. 6a was derived from Supplementary Fig. 9a-d. Supplementary Fig. 9e-h served as source data for Fig. 6b.

Supplementary Fig. 10. A possible mechanism by which plasma induces functionalization of collagen solution, leading to biofunctionalization of hydrophobic substrates. (a) Electric charge–driven changes in surface and interfacial properties of collagen solution, leading to an increase in wettability on hydrophobic surfaces. **(b)** Plasma causes changes in the local conformation and hydrogen bonding states of collagen molecules without inducing global changes in the structure of collagen molecules. **(c)** Plasma treatment induces the collagen molecules to assume a micelle-like association that leads to an increase in reactivity with hydrophobic substrates.

Description of Additional Supplementary Files

File Name: Supplementary Movie 1

Description: Time-dependent variation in a droplet of collagen solution (*left*) or plasma-treated collagen solution (*right*) on a hydrophobic cover glass. Movie shows 150-s change at a frame rate of 5 fps.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryMovie1.avi](#)