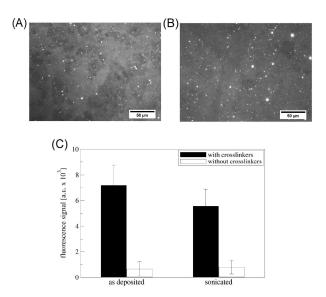**Figure 6.** (**A**,**B**) Fluorescence microscopy images of the TBA-FAM distribution on the (**A**) thin and (**B**) sonicated NDCA film; both surfaces have been activated with EDC/sNHS chemistry (with crosslinkers); (**C**) fluorescence intensity values (in greyscale) corresponding to the mean values of six areas on two different NDCA-functionalized Si_3N_4 surfaces activated with EDC/sNHS chemistry (with crosslinkers) and not activated (without crosslinkers); the error bars represent the standard deviation.

4. Discussion

In the present study, we optimized the SuMBD growth to kinetically activate NDCA molecules that can be grafted as a COOH-terminated linker to Si_3N_4 surfaces (Figure 1). The analyses of films with different thicknesses as well as sonicated NDCA films (Figures 2, 4 and 5) indicate that sonication of the samples in aqueous buffer solution removes the weakly bound molecules, exposing a homogenous NDCA monolayer covalently bound to the Si_3N_4 surface while keeping the layer's molecule structure intact. The monolayer formation most likely occurred through C–N covalent bonds between one carboxylic group of NDCA and the nitrogen atoms of the Si_3N_4 surface. The role of other chemical groups on the surface (e.g., -OH) cannot be entirely excluded. However, the chemical bond at the organic/inorganic interface is strong, most likely covalent, as supported by our findings that all weakly bounded species are removed by sonication. This in turn leads to an upward-oriented COOH group available for further attachment of biomolecules. The interlayer formation (SuMBD and sonication in water/aqueous buffer solution) is achievable in about 30 min without the use of organic solvents, which makes the entire process comparably faster and simpler with respect to conventional wet-chemical approaches to functionalize oxide-free silicon-based surfaces.

The growth behavior of NDCA films on Si_3N_4 was then systematically investigated by XPS analysis. The evolution of the XPS peak area for the substrate-specific core levels (i.e., Si 2p and N 1s) and the overlayer (i.e., C 1s) as a function of deposition time (see Figure 3) clearly indicate a characteristic Stranski–Krastanov layer-plus-island growth where the nucleation of islands begins after the complete formation and coalescence of the first two–three layers (i.e., at point T4 in Figure 3). The development of the NDCA film morphology with high-aspect-ratio islands and progressive increase in the film thickness is also confirmed by μ-FTIR mapping. The overall findings are schematically illustrated in Figure 5 (upper panel).

The absorption spectra in Figure 4 verify the suitability of NDCA as functionalizing molecule for Si_3N_4-based optical biosensors. The low absorption of the molecular layers (i.e., thick and sonicated films) in the UV-Vis range guarantees a negligible absorption of the light during excitation or fluorescence for aptamers/protein detection in optical

biosensors. Furthermore, consistently with the very low absorption of NDCA, a negligible auto-fluorescence is expected.

To demonstrate proof-of-concept for biosensing, we attached different biomolecules (TBA-FAM and AMF) to the carboxylic binding sites at the Si_3N_4 surface by coupling the carboxylic acid to the amine group of the biomolecule via carbodiimide chemistry. This kind of chemistry is widely used as a conjugation strategy to immobilize biomolecules on carboxylic activated surfaces [19,34]. The carboxylic binding sites accessible to bioreceptor molecules depend on the deposition method and on the type of substrate. Liu and co-workers, for instance, compared the surface densities of COOH groups on gold substrates using molecules that have different spatial conformations [35]. They showed that the steric hindrance of the molecule carrying the carboxylic moiety changes the final surface density of the COOH groups by four times. On a silicon nitride substrate, a density in the order of 10^{13} COOH groups/cm^2 has been reported using wet-chemical functionalization methods [36], which is in good agreement with the density of carboxylic binding sites measured in the present work. Other approaches have been used to improve the density of carboxylic groups on silicon and silicon nitride surfaces as reported by Cattaruzza et al. [16], obtaining higher surface densities. It should be noted that in the biosensor field, high surface densities are not always desirable because steric hindrance can occur in such densely packed interlayers, so that bioreceptors such as aptamers or antibodies might not have enough space to reach the proper three-dimensional conformation.

5. Conclusions

In this work, we demonstrated the possibility of functionalizing the Si_3N_4 technological surface with 2,6-naphthalene dicarboxylic acid (NDCA) through the supersonic molecular beam deposition (SuMBD), a technique working in vacuum with a high level of purity. We covalently bonded the organic molecule to the nitride surface by means of a COOH–NH interaction, leaving the second carboxylic group available for further linking. We successfully anchored two biomolecules (TBA-FAM and AMF) via carbodiimide chemistry, paving the way for the realization of optofluidic devices based on silicon nitride for the sensing of biomolecules.

The direct comparison of wet-chemistry and SuMBD experiments was beyond the scope of this research. However, we demonstrated that the advantageous properties offered by the SuMBD approach for Si_3N_4 functionalization outweigh the well-known and conventional additional processes of wet-chemical functionalization to form C–N bonded interlayers. It is predicted that SuMBD will represent an alternative route for surface functionalization and contribute to the development of future silicon nitride-based biosensors.

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References

1. Naresh, V.; Lee, N. A Review on Biosensors and Recent Development of Nanostructured Materials-Enabled Biosensors. *Sensors* **2019**, *21*, 1109. [CrossRef] [PubMed]
2. Chen, C.; Wang, J. Optical biosensors: An exhaustive and comprehensive review. *Analyst* **2020**, *145*, 1605–1628. [CrossRef] [PubMed]
3. Wu, P.; Hogrebe, P.; Grainger, D.W. DNA and protein microarray printing on silicon nitride waveguide surfaces. *Biosens. Bioelectron.* **2006**, *21*, 1252–1263. [CrossRef] [PubMed]
4. Aparicio, F.J.; Froner, E.; Rigo, E.; Gandolfi, D.; Scarpa, M.; Han, B.; Ghulinyan, M.; Pucker, G.; Pavesi, L. Silicon oxynitride waveguides as evanescent-field-based fluorescent biosensors. *J. Phys. D Appl. Phys.* **2014**, *47*, 405401. [CrossRef]
5. Muellner, P.; Melnik, E.; Koppitsch, G.; Kraft, J.; Schrank, F.; Hainberger, R. CMOS-compatible Si3N4 waveguides for optical biosensing. *Procedia Eng.* **2015**, *120*, 578–581. [CrossRef]
6. Bahri, M.; Baraket, A.; Zine, N.; Ben Ali, M.; Bausells, J.; Errachid, A. Capacitance electrochemical biosensor based on silicon nitride transducer for TNF-α cytokine detection in artificial human saliva: Heart failure (HF). *Talanta* **2020**, *209*, 120501. [CrossRef]
7. Du, X.; Lee, S.S.; Blugan, G.; Ferguson, S.J. Silicon Nitride as a Biomedical Material: An Overview. *Int. J. Mol. Sci.* **2022**, *23*, 6551. [CrossRef]
8. Chatterjee, S.; Saxena, M.; Padmanabhan, D.; Jayachandra, M.; Pandya, H.J. Futuristic medical implants using bioresorbable materials and devices. *Biosens. Bioelectron.* **2019**, *142*, 111489. [CrossRef]
9. Nitkowski, A.; Baeumner, A.; Lipson, M. On-chip spectrophotometry for bioanalysis using nanophotonic devices. *Biomedical Opt. Express* **2011**, *2*, 271–277. [CrossRef]
10. Neutens, P.; Jansen, R.; Woronoff, G.; Rutowska, M.; Hosseini, N.; Buja, F.; Humbert, A.; Colle, F.; Stakenborg, T.; Van Roy, W. Waveguide-based absorption measurement system for visible wavelength applications. *Biomed. Opt. Express* **2021**, *12*, 2041–2053. [CrossRef]
11. Porcel, M.A.G.; Hinojosa, A.; Jans, H.; Stassen, A.; Goyvaerts, J.; Geuzebroek, D.; Geiselmann, M.; Dominguez, C.; Artundo, I. Silicon nitride photonic integration for visible light applications. *Opt. Laser Technol.* **2019**, *112*, 299–306. [CrossRef]
12. Stine, R.; Cole, C.L.; Ainslie, K.M.; Mulvaney, S.P.; Whitman, L.J. Formation of primary amines on silicon nitride surfaces: A direct, plasma-based pathway to functionalization. *Langmuir* **2007**, *23*, 4400–4404. [CrossRef]
13. Rosso, M.; Giesbers, M.; Arafat, A.; Schroën, K.; Zuilhof, H. Covalently attached organic monolayers on SiC and SixN 4 surfaces: Formation using UV light at room temperature. *Langmuir* **2009**, *25*, 2172–2180. [CrossRef] [PubMed]
14. Diao, J.; Ren, D.; Engstrom, J.R.; Lee, K.H. A surface modification strategy on silicon nitride for developing biosensors. *Anal. Biochem.* **2005**, *343*, 322–328. [CrossRef]
15. Antoniou, M.; Tsounidi, D.; Petrou, P.S.; Beltsios, K.G.; Kakabakos, S.E. Functionalization of silicon dioxide and silicon nitride surfaces with aminosilanes for optical biosensing applications. *Med. Devices Sens.* **2020**, *3*, e10072. [CrossRef]
16. Cattaruzza, F.; Cricenti, A.; Flamini, A.; Girasole, M.; Longo, G.; Mezzi, A.; Prosperi, T. Carboxylic acid terminated monolayer formation on crystalline silicon and silicon nitride surfaces. A surface coverage determination with a fluorescent probe in solution. *J. Mater. Chem.* **2004**, *14*, 1461–1468. [CrossRef]
17. Arafat, A.; Schroën, K.; De Smet, L.C.P.M.; Sudhölter, E.J.R.; Zuilhof, H. Tailor-made functionalization of silicon nitride surfaces. *J. Am. Chem. Soc.* **2004**, *126*, 8600–8601. [CrossRef]
18. Arafat, A.; Giesbers, M.; Rosso, M.; Sudhölter, E.J.R.; Schroën, K.; White, R.G.; Li, Y.; Linford, M.R.; Han, Z. Covalent biofunctionalization of silicon nitride surfaces. *Langmuir* **2007**, *23*, 6233–6244. [CrossRef]
19. Henriksson, A.; Neubauer, P.; Birkholz, M. Functionalization of Oxide-Free Silicon Surfaces for Biosensing Applications. *Adv. Mater. Interfaces* **2021**, *8*, 2100927. [CrossRef]
20. Iannotta, S.; Toccoli, T. Supersonic Molecular Beam Growth of Thin Films of Organic Materials: A Novel Approach to Controlling the Structure, Morphology, and Functional Properties. *J. Polym. Sci. Part B Polym. Phys.* **2003**, *41*, 2501–2521. [CrossRef]
21. Wu, Y.; Toccoli, T.; Koch, N.; Iacob, E.; Pallaoro, A.; Rudolf, P.; Iannotta, S. Controlling the early stages of pentacene growth by supersonic molecular beam deposition. *Phys. Rev. Lett.* **2007**, *98*, 076601. [CrossRef] [PubMed]
22. Gottardi, S.; Toccoli, T.; Wu, Y.; Iannotta, S.; Rudolf, P. Growth dynamics in supersonic molecular beam deposition of pentacene sub-monolayers on SiO$_2$. *Chem. Commun.* **2014**, *50*, 7694–7697. [CrossRef] [PubMed]
23. Verucchi, R.; Aversa, L.; Nardi, M.V.; Taioli, S.; Beccara, S.A.; Alfè, D.; Nasi, L.; Rossi, F.; Salviati, G.; Iannotta, S. Epitaxy of nanocrystalline silicon carbide on Si(111) at room temperature. *J. Am. Chem. Soc.* **2012**, *134*, 17400–17403. [CrossRef]
24. Daldosso, N.; Melchiorri, M.; Riboli, F.; Sbrana, F.; Pavesi, L.; Pucker, G.; Kompocholis, C.; Crivellari, M.; Bellutti, P.; Lui, A. Fabrication and optical characterization of thin two-dimensional Si 3N4 waveguides. *Mater. Sci. Semicond. Process.* **2004**, *7*, 453–458. [CrossRef]
25. Melchiorri, M.; Daldosso, N.; Sbrana, F.; Pavesi, L.; Pucker, G.; Kompocholis, C.; Bellutti, P.; Lui, A. Propagation losses of silicon nitride waveguides in the near-infrared range. *Appl. Phys. Lett.* **2005**, *86*, 121111. [CrossRef]
26. Schneider, C.A.; Rasband, W.S.; Eliceiri, K.W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **2012**, *9*, 671–675. [CrossRef]
27. Schöll, A.; Zou, Y.; Jung, M.; Schmidt, T.; Fink, R.; Umbach, E. Line shapes and satellites in high-resolution x-ray photoelectron spectra of large π-conjugated organic molecules. *J. Chem. Phys.* **2004**, *121*, 10260–10267. [CrossRef]

28. Kong, X.; Castarède, D.; Boucly, A.; Artiglia, L.; Ammann, M.; Bartels-Rausch, T.; Thomson, E.S.; Pettersson, J.B.C. Reversibly Physisorbed and Chemisorbed Water on Carboxylic Salt Surfaces under Atmospheric Conditions. *J. Phys. Chem. C* **2020**, *124*, 5263–5269. [CrossRef]

29. Hall, S.J.; Klein, B.P.; Maurer, R.J. Characterizing Molecule-Metal Surface Chemistry with Ab Initio Simulation of X-ray Absorption and Photoemission Spectra. *J. Phys. Chem. C* **2023**, *127*, 1870–1880. [CrossRef]

30. Nardi, M.; Verucchi, R.; Aversa, L.; Casarin, M.; Vittadini, A.; Mahne, N.; Giglia, A.; Nannarone, S.; Iannotta, S. Electronic properties of tetrakis(pentafluorophenyl)porphyrin. *New J. Chem.* **2013**, *37*, 1036. [CrossRef]

31. Rai, D.K.; Solanki, C.S.; Kavaipatti, B.R. Growth of silicon nitride by nitridation of amorphous silicon at low temperature in hot-wire CVD. *Mater. Sci. Semicond. Process.* **2017**, *67*, 46–54. [CrossRef]

32. Frank, F.C.; van der Merwe, J.H. One-dimensiomnal dislocations I. Static theory. *Proc. R. Soc. A* **1949**, *198*, 205–216.

33. Finnie, K.S.; Bartlett, J.R.; Woolfrey, J.L. Vibrational spectroscopic study of the coordination of (2,2′-bipyridyl-4,4′-dicarboxylic acid)ruthenium(II) complexes to the surface of nanocrystalline titania. *Langmuir* **1998**, *14*, 2741–2749. [CrossRef]

34. Hermanson, G.T. *Bioconjugate Techniques*; Academic Press: Amsterdam, The Netherlands, 2013; ISBN 978-0-12-382239-0.

35. Liu, J.; Shekhah, O.; Stammer, X.; Arslan, H.K.; Liu, B.; Schüpbach, B.; Terfort, A.; Wöll, C. Deposition of metal-organic frameworks by liquid-phase epitaxy: The influence of substrate functional group density on film orientation. *Materials* **2012**, *5*, 1581–1592. [CrossRef]

36. Masuda, Y.; Inami, W.; Miyakawa, A.; Kawata, Y. Cell culture on hydrophilicity-controlled silicon nitride surfaces. *World J. Microbiol. Biotechnol.* **2015**, *31*, 1977–1982. [CrossRef] [PubMed]