

ACCEPTED VERSION

A. Bachhuka, R. Madathiparambil Visalakshan, C. S. Law, A. Santos, H. Ebendorff-Heidepriem, S. Karnati, and K. Vasilev

Modulation of macrophages differentiation by nanoscale-engineered geometric and chemical features

ACS Applied Bio Materials, 2020; 3(3):1496-1505

This document is the Accepted Manuscript version of a Published Work that appeared in final form in ACS Applied Bio Materials, copyright © 2020 American Chemical Society after peer review and technical editing by the publisher. To access the final edited and published work see <http://dx.doi.org/10.1021/acsabm.9b01125>

PERMISSIONS

<http://pubs.acs.org/page/4authors/jpa/index.html>

The new agreement specifically addresses what authors can do with different versions of their manuscript – e.g. use in theses and collections, teaching and training, conference presentations, sharing with colleagues, and posting on websites and repositories. The terms under which these uses can occur are clearly identified to prevent misunderstandings that could jeopardize final publication of a manuscript (**Section II, Permitted Uses by Authors**).

[Easy Reference User Guide](#)

7. Posting Accepted and Published Works on Websites and Repositories: A digital file of the Accepted Work and/or the Published Work may be made publicly available on websites or repositories (e.g. the Author's personal website, preprint servers, university networks or primary employer's institutional websites, third party institutional or subject-based repositories, and conference websites that feature presentations by the Author(s) based on the Accepted and/or the Published Work) under the following conditions:

- It is mandated by the Author(s)' funding agency, primary employer, or, in the case of Author(s) employed in academia, university administration.
- If the mandated public availability of the Accepted Manuscript is sooner than 12 months after online publication of the Published Work, a waiver from the relevant institutional policy should be sought. If a waiver cannot be obtained, the Author(s) may sponsor the immediate availability of the final Published Work through participation in the ACS AuthorChoice program—for information about this program see <http://pubs.acs.org/page/policy/authorchoice/index.html>.
- If the mandated public availability of the Accepted Manuscript is not sooner than 12 months after online publication of the Published Work, the Accepted Manuscript may be posted to the mandated website or repository. The following notice should be included at the time of posting, or the posting amended as appropriate:
"This document is the Accepted Manuscript version of a Published Work that appeared in final form in [JournalTitle], copyright © American Chemical Society after peer review and technical editing by the publisher. To access the final edited and published work see [insert ACS Articles on Request author-directed link to Published Work, see <http://pubs.acs.org/page/policy/articlesonrequest/index.html>]."
• The posting must be for non-commercial purposes and not violate the ACS' "Ethical Guidelines to Publication of Chemical Research" (see <http://pubs.acs.org/ethics>).
• Regardless of any mandated public availability date of a digital file of the final Published Work, Author(s) may make this file available only via the ACS AuthorChoice Program. For more information, see <http://pubs.acs.org/page/policy/authorchoice/index.html>.

24 March 2021

<http://hdl.handle.net/2440/124848>

Modulation of macrophages differentiation by nanoscale-engineered geometric and chemical features

Akash Bachhuka, Rahul Madathiparambil Visalakshan, Cheryl Suwen Law, Abel Santos, Heike Ebdorff-Heidepriem, Srikanth Karnati, and Krasimir Vasilev

ACS Appl. Bio Mater., **Just Accepted Manuscript** • DOI: 10.1021/acsabm.9b01125 • Publication Date (Web): 16 Feb 2020

Downloaded from pubs.acs.org on February 23, 2020

Just Accepted

“Just Accepted” manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides “Just Accepted” as a service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. “Just Accepted” manuscripts appear in full in PDF format accompanied by an HTML abstract. “Just Accepted” manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are citable by the Digital Object Identifier (DOI®). “Just Accepted” is an optional service offered to authors. Therefore, the “Just Accepted” Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the “Just Accepted” Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these “Just Accepted” manuscripts.

1
2
3
4 Modulation of macrophages differentiation by
5
6
7
8
9
10
11 nanoscale-engineered geometric and
12
13
14
15
16
17 chemical features
18
19
20
21
22

23 *A. Bachhuka*^{1,2#*}, *R. Madathiparambil Visalakshan*^{3#}, *C.S. Law*^{1,2,4}, *A. Santos*^{1,2,4}, *H.*

24
25
26
27 *Ebendorff-Heidepriem*^{1,2}, *S. Karnat*⁵, *K. Vasilev*^{3, 6*}
28
29

30
31 *1 ARC Center of Excellence for Nanoscale BioPhotonics (CNBP), The University of*
32
33
34 *Adelaide, SA 5005, Adelaide, Australia*
35
36

37
38 *2 Institute for Photonics and Advanced Sensing (IPAS), The University of Adelaide,*
39
40
41 *SA 5005, Adelaide, Australia.*
42
43

44
45 *3 Future Industries Institute, University of South Australia, Mawson lakes Campus,*
46
47
48 *SA, 5095, Australia.*
49

50
51
52 *4 School of Chemical Engineering, University of Adelaide, Engineering North Building,*
53
54
55 *SA 5005 Adelaide, Australia.*
56
57
58
59
60

1
2
3
4 *5 Institute for Anatomy and Cell Biology, Julius Maximilians University,*
5
6
7 *Koellikerstrasse 6, Wuerzburg, 97070, Germany.*
8
9

10
11 *6 School of Engineering, University of South Australia, Mawson lakes*
12
13
14 *Campus, SA, 5095, Australia.*
15
16

17
18 *# Authors with equal contribution*
19

20
21
22 **Corresponding author email address: akash.bachhuka@adelaide.edu.au*
23

24
25 **Corresponding author email address: Krasimir.vasilev@unisa.edu.au*
26
27

28
29 KEYWORDS: plasma polymerization, nanoporous substrates, surface chemistry, pro-
30
31
32 inflammatory cytokines, anti-inflammatory cytokines, foreign body response, wound
33
34
35 healing.
36
37

38
39
40 ABSTRACT
41

42
43
44 Macrophages differentiation into M1 (inflammatory) and M2 (healing) phenotypes
45
46
47 plays a vital role in determining the fate of biomaterials. The biophysical properties of
48
49
50 the extra-cellular matrix are known to affect macrophage behavior. Mimicking these
51
52
53 special biophysical properties of the extra-cellular matrix have led to increasing
54
55
56 interest in biomaterial constructs with tailor-engineered surface nanotopographical
57
58
59
60

1
2
3 and chemical properties. However, significant gap of knowledge exists in the role
4
5
6
7 played by the combinational effect of surface nanotopography and chemistry. To
8
9
10 address this gap, we have fabricated nanoporous surfaces of controlled pore size (30,
11
12
13 65 and 200 nm) and lateral spacing with uniform outermost surface chemistry tailored
14
15
16 with amines (NH_2), carboxyl (COOH^-) and hydrocarbon (CH_3^-) functionalities. We
17
18
19
20
21 show that the combinatory effects of surface properties can direct the differentiation of
22
23
24 macrophages to the pro-healing M2 phenotype. This is most evident on the surface
25
26
27 containing featuring nanopores of 200 nm and $-\text{COOH}$ functionality. Overall, the
28
29
30 concentration of pro-inflammatory cytokines significantly decreases while the
31
32
33 concentration of anti-inflammatory cytokines increases many folds on
34
35
36
37
38 nanotopographically, and chemically modified surfaces compared to their planar
39
40
41 counterparts. Our data provides pioneering knowledge that could provide pathways to
42
43
44
45 tuning inflammatory and foreign body responses and instruct the design of tailor-
46
47
48
49 engineered biomaterial implants to enable better clinical outcomes.
50
51
52
53
54
55
56
57
58
59
60

INTRODUCTION

The biggest performance challenge that biomaterial implants face is modulation of the host immune response. Immediately upon implantation, adsorption and desorption of different proteins occur on the biomaterial's surface, a phenomenon known as the Vroman effect¹⁻². This governs the subsequent biological phenomena underlying binding and activation of waves of innate immune effector cells such as neutrophils, macrophages, dendritic cells, mast cells, granulocytes and natural killer cells³⁻⁸. Of all these, macrophages are an important class of immune cells which attempt to eliminate the foreign body (i.e. biomaterial implant) by fusing among themselves to form foreign body giant cells (FBGC). Macrophages are a critical component of the host immune response, both to implants and microorganisms, through their phagocytic activities⁹. Macrophages are considered as 'plastic' cells, being categorized into 'M1' and 'M2' phenotypes based on their respective roles^{8, 10-12}. M1-activated macrophages are key elements in inflammation and responsible for 'cleaning the site' by expressing pro-inflammatory cytokines such as tumor necrosis factor (TNF α) and interleukin (IL6 and

1
2
3
4 IL1 β). Conversely, the M2-activated macrophages produce anti-inflammatory
5
6
7 cytokines (Arginase, IL10 and IL1RA) and are involved in wound healing and
8
9
10 remodeling.

11
12
13
14 Recent studies by our group and others have shown that macrophage adhesion,
15
16
17 phagocytic activity and cytokine production can be modulated by using biomaterial
18
19
20 surface properties such as chemistry and nanotopography. These engineering tools
21
22
23
24
25 can be effectively used to modulate inflammatory responses by biomaterial surfaces
26
27
28 with tailored properties¹³⁻¹⁷. This provides exciting opportunity for the rational design
29
30
31
32 of biomedical constructs to modulate and elicit desired immunological responses. For
33
34
35
36 example, our work utilizing 'hill-like' nanotopography of 16 nm, 38 nm and 68 nm
37
38
39 demonstrated that the scale of nanofeature significantly affected immune cells
40
41
42 attachment and expression of inflammatory markers¹³⁻¹⁵. The effect of
43
44
45
46 nanotopography was further modulated by the outermost surface chemistry, revealing
47
48
49 a complex picture of mutually dependent parameters but also an opportunity to
50
51
52
53 harness surface properties to design and guide inflammatory responses to
54
55
56 biomaterials. We have also recently reported that 'hill-like' nanotopography induced
57
58
59 conformational changes to adsorbed fibrinogen leading to unfolding and presentation
60

1
2
3 of normally hidden peptide sequences that activate the MAC-1 receptor of
4
5
6
7 inflammatory cells¹⁷.
8
9

10
11 In this work, we further expand the knowledge in the field and reveal the combinational
12
13 role of nanopores and surface chemistry on modulating immune cell responses. As
14
15 model substrata, we employed anodic alumina (NAA) which provides a nanoporous
16
17
18 surface with hexagonally distributed nanopores. The geometric features of these NAA
19
20
21 substrates can be precisely controlled by altering the fabrication conditions. NAA-
22
23
24 based implants are not envisaged for developing brain implants due to potential
25
26
27 leaching of aluminium ions under physiological conditions. However, NAA is
28
29
30
31
32
33
34
35
36
37
38
39 orthopaedic and dental implants^{18,19}. Many studies have reported on the use of NAA-
40
41
42
43 coated implants (e.g. orthopaedic, dental, coronary, etc.) and immunoisolation,
44
45
46 showing reduced leaching of aluminium ions under physiological conditions²⁰. NAA
47
48
49 surfaces have also been engineered as active drug-releasing coatings in orthopaedic,
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000

1
2
3 immunoisolation studies performed onto NAA surfaces suggest that this nanoporous
4
5
6
7 material does not generate significant complement activation. However, *in vivo*
8
9
10 transient inflammatory response was observed for unmodified and PEG-functionalized
11
12
13
14 NAA surfaces upon implantation into the peritoneal cavity of rats²⁰. Reduction in
15
16
17 granulation along with the existence of blood vessels in the tissue surrounding the
18
19
20
21 NAA implant indicated complex inflammatory consequences that require further
22
23
24 elucidation. Furthermore, the interplay of nanotopography and surface chemistry in
25
26
27
28 macrophage differentiation has not been comprehensively investigated. To unravel
29
30
31 these phenomena, the outermost surface chemistry of NAA with varying porous
32
33
34 structure was tailored by a thin layer of functional polymers deposited by plasma. A
35
36
37
38 key benefit of plasma polymerization is that, it generates coatings of desired
39
40
41
42 physicochemical properties in an arbitrary substrate, without requiring any pre-
43
44
45 modification of the surface²⁵ compared to techniques such as LbL or SAMs²⁶. The
46
47
48 method consists of a single step, occurring within minutes and does not require
49
50
51
52 solvents which provides benefit in terms of time costs²⁷⁻³⁰. Moreover, these coatings
53
54
55
56 can also be deposited on complex structures including micro and nano particles^{16, 31-}
57
58
59 ³⁴. Immune responses were evaluated on these nanotopographically and chemically
60

1
2
3
4 modified surfaces by measuring the expression of pro- and anti-inflammatory
5
6
7 cytokines from macrophages.
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53

54 MATERIALS AND METHODS

55
56
57
58
59 Materials:
60

1
2
3 Aluminium foils (99.9997% purity and 0.32mm thick) were purchased from Goodfellow
4
5
6 Cambridge Ltd. (UK). Oxalic acid ($\text{H}_2\text{C}_2\text{O}_4$), perchloric acid (HClO_4) and chromic acid
7
8
9 (H_2CrO_4), were supplied by Sigma-Aldrich (Australia). Ethanol ($\text{C}_2\text{H}_5\text{OH}$, EtOH),
10
11
12 sulfuric acid (H_2SO_4) and phosphoric acid (H_3PO_4) were purchased from ChemSupply
13
14
15
16
17 (Australia). Ultrapure Milli-Q® water (18.2 mΩ·cm) was utilized to prepare all aqueous
18
19
20
21 solutions.

22 23 24 25 Fabrication of Nanoporous Anodic Alumina:

26
27
28 Aluminum substrates were cleaned under sonication in a bath of EtOH and Milli-Q®
29
30
31 water for 15 min each before anodization, and dried under air stream. The surface of
32
33
34 cleaned Al substrates was electropolished in an electrolyte of HClO_4 and EtOH 1:4
35
36
37
38 (v/v) at 20 V and 5 °C for 3 min. This process was performed in an electrochemical
39
40
41 reactor with a circular window of ~1 cm in diameter. Three types of nanoporous anodic
42
43
44 alumina (NAA) substrates with tuned geometric features of nanopores were fabricated
45
46
47 by two-step anodization³⁵⁻³⁸: i) NAA produced in sulfuric acid (NAA-Su, 30 nm), ii) NAA
48
49
50 fabricated in oxalic acid (NAA-Ox, 65 nm), and iii) NAA anodized in phosphoric acid
51
52
53
54
55
56 (NAA-Ph, 200 nm). The first anodization step was performed for 20 h in 0.3 M sulfuric
57
58
59
60

1
2
3 acid at 6 °C for NAA-Su, 0.3 M oxalic acid at 6 °C for NAA-Ox, and 0.1 M phosphoric
4
5
6
7 acid for NAA-Ph at 1 °C, with anodization voltages of 25, 40 and 195 V, respectively.
8
9

10 The resulting NAA films with disordered nanopores at the top were selectively
11
12 removed by wet chemical etching in 0.2 M H₂CrO₄ and 0.4 M H₃PO₄ at 70 °C for 3 h.
13
14
15

16
17 Then, we performed the second anodization step under the same conditions as during
18
19 the first step but for 2 h. The final nanopore size in the NAA films was precisely tuned
20
21 by by wet chemical etching in H₃PO₄ 5 *wt*% at 35 °C for 8, 18 and 30 min for 35 nm,
22
23
24
25
26
27
28 65 nm and 200 nm samples, respectively.
29
30

31 32 Plasma Polymerization:

33
34
35 A plasma reactor with a 13.56 MHz generator was utilized to modify NAA substrates
36
37 with desired surface chemistry²⁷. Nanoporous membranes were cleaned under oxygen
38
39 plasma for 2 minutes at a power of 50W. Methyl oxazoline, acrylic acid and octadiene
40
41
42
43
44
45
46 were utilized to overcoat the surface of NAA substrates with a 5nm thin layer of plasma
47
48
49 polymer coating. Surface coatings of methyl oxazoline, acrylic acid and octadiene
50
51
52
53 were deposited using a power of 40, 10 and 20 W, respectively, while the deposition
54
55
56
57 time was kept constant at 20 s for all three monomers.
58
59
60

1
2
3 Scanning Electron Microscopy:
4
5

6
7 The geometrical features of the NAA substrates were established by field emission
8
9
10 gun scanning electron microscopy (FEG-SEM FEI Quanta 450). Image J was utilized
11
12
13
14 for processing FEG-SEM images.
15

16
17
18 X-ray photoelectron spectroscopy:
19
20

21
22 Elemental composition of the plasma surface coatings deposited onto the surface of
23
24
25 the NAA substrates were determined using XPS. A Spec SAGE XPS
26
27
28 spectrophotometer with a monochromatic Mg radiation source was operated at 10 kV
29
30
31 and 20 mA to record all XPS spectra over 0-1000eV at a pass energy of 100eV and
32
33
34
35 resolution of 0.5 eV. Survey spectra were then utilized to quantify atomic percentage
36
37
38
39 of the elements present in the polymers. Neutral C1s carbon peak at binding energy
40
41
42 (BE) of 285.0 eV was used as a reference to correct all other BE. All spectra were
43
44
45
46 quantified using casaXPS.
47
48
49

50 Ellipsometer:
51
52

53
54 Silicon wafers were kept adjacent to the NAA substrates and plasma coated using the
55
56
57 same parameters. A variable angle spectroscopic ellipsometer (J. A. Woollam Co.
58
59
60

1
2
3 Inc.) was used to measure the thickness of the polymer coatings. Reference silicon
4
5
6
7 wafer was used for calibration and then all measurements were performed over a
8
9
10 wavelength range of 250 to 1100 nm at 10nm increment at different angles from 65°
11
12
13 to 75° at an interval of 5°. The data obtained was quantified using Cauchy model. 3
14
15
16
17 measurements per sample were performed to obtain the average thickness, which
18
19
20
21 were reported to have less than 10% experimental error.
22
23

24 25 Cell Culture:

26
27
28 THP-1 cells (Human monocytes) were grown in RPMI 1640 (Sigma Aldrich) with 1%
29
30
31 (v/v) penicillin/streptomycin (Life Technologies) and 10 % fetal bovine serum (FBS,
32
33
34 Thermo Scientific) and were then used for immune studies. An incubator set at 37 °C
35
36
37
38 containing 5% CO₂ was used for maintaining cells and growth media was changed at
39
40
41
42 80% confluency (i.e. every 3 days).
43
44
45

46 47 Inflammatory response of macrophage:

48
49
50 Phorbol-12-myristate 13-acetate (PMA, 100ng/ml) was used to differentiate THP-1
51
52
53 cells into dTHP-1 (macrophages), according to the previously reported protocol³⁹⁻⁴⁰.
54
55
56
57 Cells were treated for 48 h with media containing PMA and for another 24 h with fresh
58
59
60

1
2
3
4 media without PMA. Differentiated dTHP-1 macrophage cells were seeded on
5
6
7 unmodified and modified NAA substrates at a density of 1×10^5 cells ml^{-1} and grown
8
9
10 overnight for cell attachment. Once the cells were attached, the media was changed
11
12
13 with fresh media containing $1\mu\text{g ml}^{-1}$ LPS (lipopolysaccharide) to activate the
14
15
16 macrophages. Cells were exposed with LPS for further 6 h and conditioned media
17
18
19 were collected for quantification of cytokine production⁴¹. After collecting the media,
20
21
22 macrophage cell counts were performed using trypsin and hemocytometer to quantify
23
24
25 the number of cells that produced cytokines on each surface. Pro- and anti-
26
27
28 inflammatory cytokines [IL-12p70, TNF- α , IL-6, IL-1 β , IL-12p40, IL-23, IFN- γ , IP-10,
29
30
31 IL-4, IL-10, Arginase, and TARC] were quantified using LEGENDplex human
32
33
34 macrophage/microglia Panel (13-plex) and ELISA kits (BioLegend, San Diego, CA,
35
36
37 USA) following the manufacturer's instructions.
38
39
40
41
42
43
44
45

46 Statistical Analysis:

47
48
49 Graph Pad prism 8 was used to quantify all statistical analysis using a 1-way ANOVA
50
51
52 with Dunnett's multiple comparison test. The data obtained from all measurements
53
54
55 (n=9) was presented as mean \pm standard error mean (SEM).
56
57
58
59
60

RESULTS

Fabrication of NAA substrates having defined porous structure and outermost surface chemistry

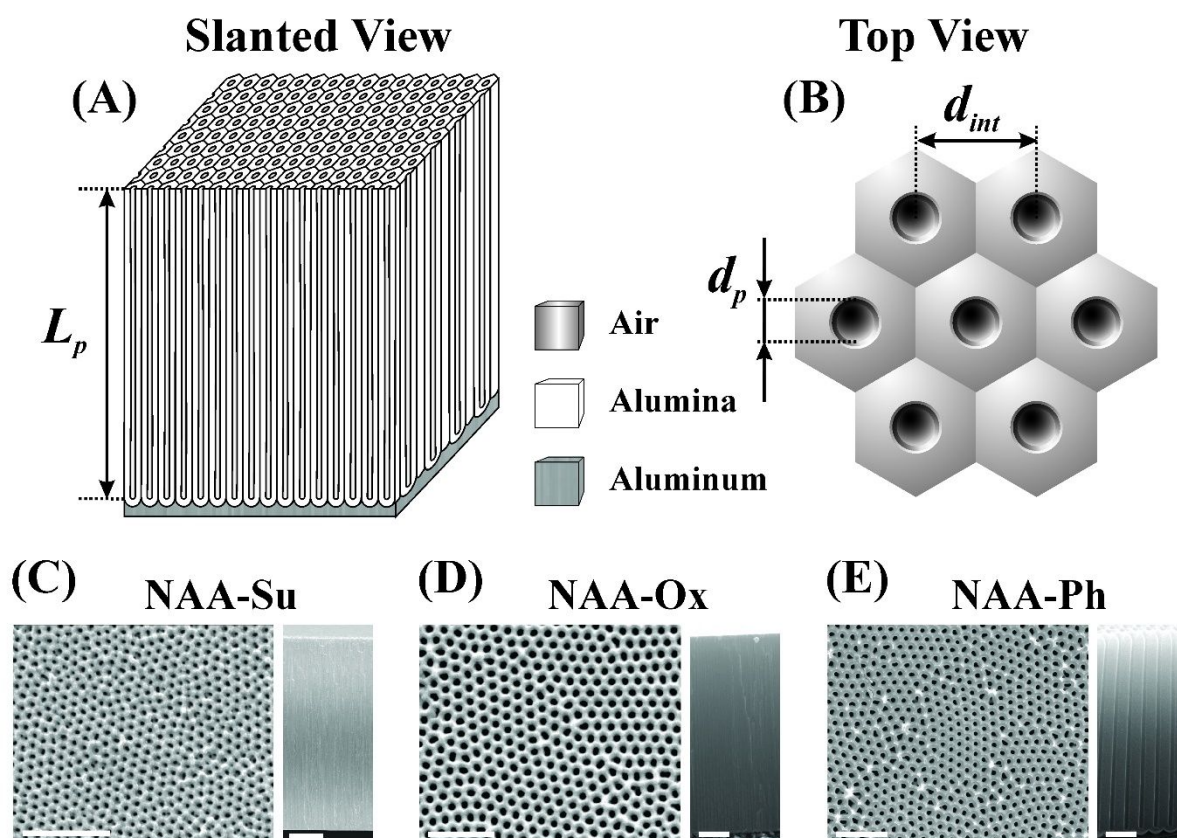


Figure 1: Geometric parameters of NAA substrates produced by two-step anodization.

A,B) Slanted and top view schematics of a NAA substrate with details of geometric parameters (i.e. nanopore diameter d_p ; nanopore length L_p ; interpore distance d_{int}). C-

E) Top (left) and cross-sectional (right) FEG-SEM images of NAA produced in sulfuric (scale bar (left) = 500 nm; scale bar (right) = 2 μ m), oxalic acid (scale bar (left) = 500

1
2
3 nm; scale bar (right) = 1 μm), and phosphoric acid (scale bar (left) = 2.5 μm ; scale bar
4
5
6
7 (right) = 1 μm), respectively. The geometric parameters of NAA films (i.e. nanopore
8
9
10 diameter d_p ; nanopore length L_p ; interpore distance d_{int} ; and pore density r_p ; **Figures**
11
12
13
14 **1A and B**) were measured through FEG-SEM image analysis. **Figures 1C–E** show
15
16
17 representative FEG-SEM images of the cross-section and top surface of NAA films
18
19
20 fabricated in this study^{35–38}. **Figures 1C–E** show cross-sectional FEG-SEM images of
21
22
23 NAA films with straight cylindrical nanopores grown perpendicularly to the underlying
24
25
26 aluminum substrate. These nanopores feature a closed oxide barrier layer at their
27
28
29 bottom (**Figure 1A**). In average, the nanopore length of NAA-Su, NAA-Ox and NAA-
30
31
32 Ph substrates under the fabrication conditions used in our study were $L_p = 13.1 \pm 0.5$,
33
34
35 6.1 ± 0.4 and 7.5 ± 0.3 μm , respectively. The top surface of NAA substrates shows an
36
37
38 array of nanopores of uniform size and distribution arranged in a self-organized
39
40
41
42 hexagonal pattern (**Figures 1C–E**). The average nanopore diameter and interpore
43
44
45 distance for NAA-Su, NAA-Ox and NAA-Ph substrates were $d_p = 30 \pm 2$, 65 ± 4 and
46
47
48 200 ± 4 nm and $d_{int} = 66 \pm 3$, 105 ± 5 and 449 ± 24 nm, respectively. The pore densities
49
50
51 (i.e. number of nanopores per unit area) were $r_p = 2.65 \times 10^{11}$, 1.05×10^{11} and $5.73 \times$
52
53
54
55
56
57
58
59 10^9 cm^{-2} for NAA-Su, NAA-Ox and NAA-Ph substrates, respectively.
60

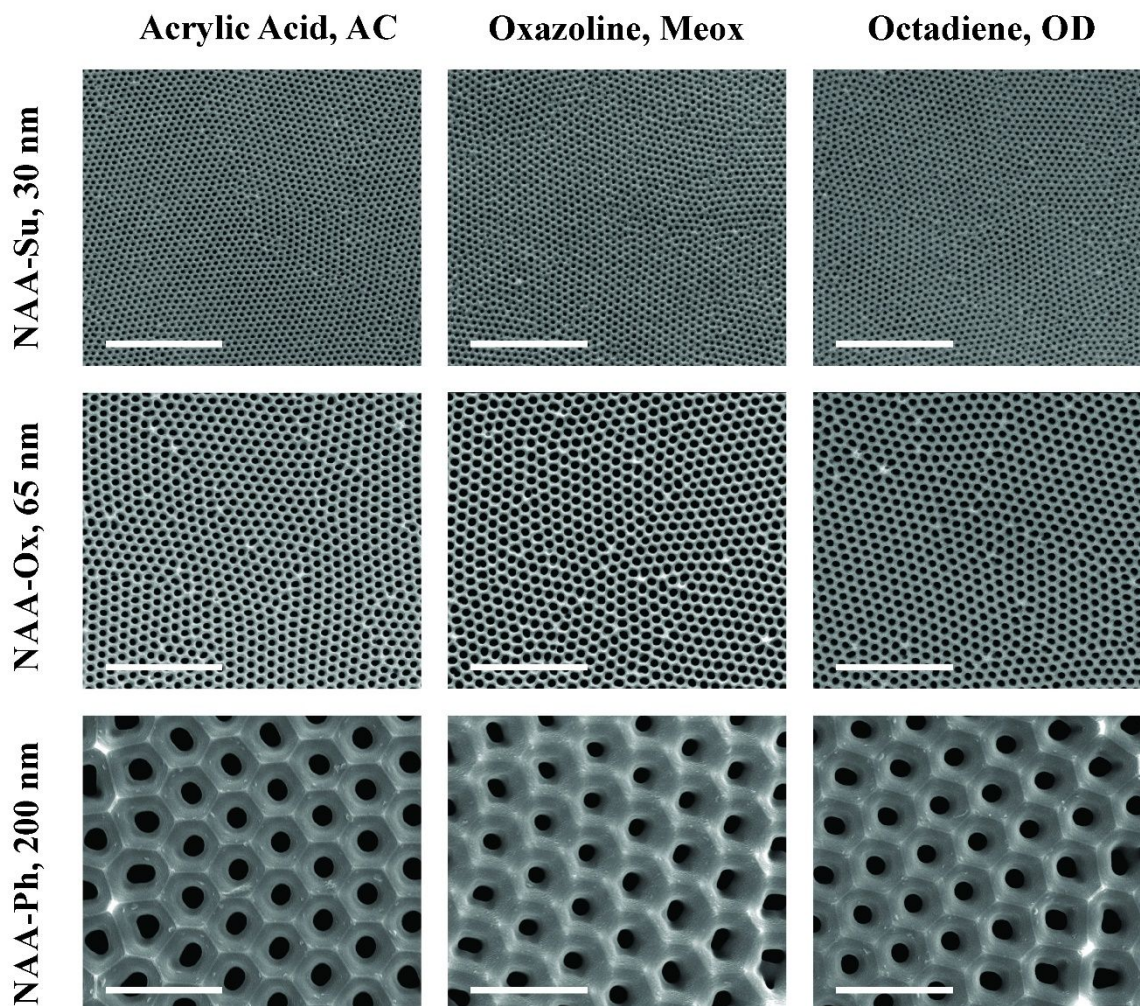


Figure 2: FEG-SEM image of nanoporous anodic alumina with three different pore sizes, 30 nm, 65 nm and 200 nm (scale bars = 1 μ m) overcoated with three different chemistries (acrylic acid, methyl oxazoline and octadiene).

NAA substrates with desired outermost surface chemistry was obtained by overcoating 5 nm thick layer of plasma polymers obtained from different monomers

1
2
3 such as acrylic acid, methyl oxazoline and octadiene (ACpp, Meoxpp, ODpp). These
4
5
6
7 surface coatings were chosen as they represent chemical compositions consistent
8
9
10 with that of biological matter such as in amino acids, extra-cellular matrix and proteins
11
12
13 (i.e. COOH- (ACpp), NH₂- (Meoxpp) and CH₃- (ODpp)⁴²⁻⁴³. All these coatings have
14
15
16
17 negative surface charges in aqueous medium at physiological pH = 7.4 as Meoxpp
18
19
20 and ODpp coatings are slightly negatively charged -18 mV and -19 mV, respectively,
21
22
23
24 whereas the ACpp coatings had the highest negative charge of -28 mV (**Figure S1**)⁴⁴⁻
25
26
27
28 ⁴⁵. The different chemistries of the coatings result in different wetting characteristics,
29
30
31 as indicated by water contact angles of 35° for ACpp, 60° for Meoxpp and 85° for
32
33
34 ODpp (**Figure S2**)^{17, 46}. The thickness of the plasma polymer films was tailored to be
35
36
37
38 of 5 nm, in order to preserve as much as possible the original nanoporous structure.
39
40
41 We know from our published work that plasma polymer films of 5 nm and above are
42
43
44
45 continuous and pinhole-free, allowing us to preserve the nanotopography generated
46
47
48
49 by the NAA substrates and thus study the combinational effect of nanotopography and
50
51
52 surface chemistry^{25, 47}. FEG-SEM images of overcoated NAA substrates are shown in
53
54
55
56 **Figure 2**. The images demonstrate that the nanopores retain their original shape and
57
58
59
60

the surface morphology (i.e. nanopore diameter) is not affected by the outermost surface chemistry.

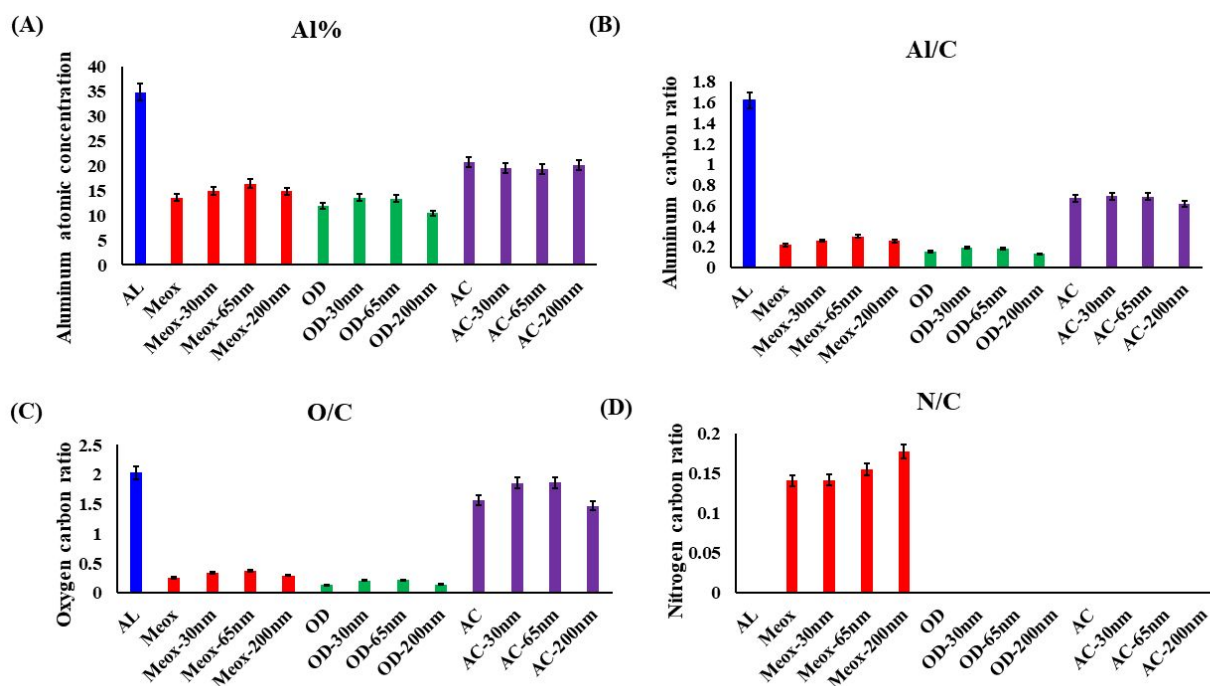
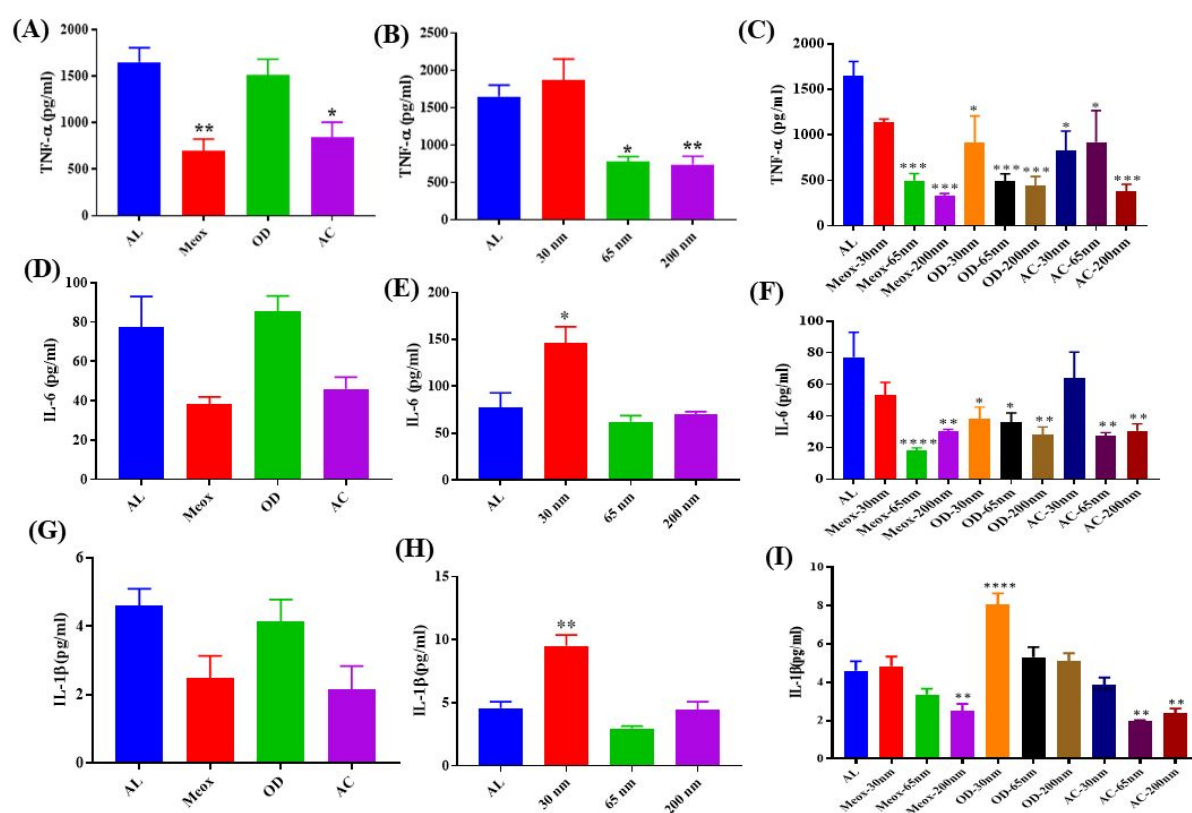


Figure 3: Surface chemical composition determined by XPS. Atomic percentage of element of interest on NAA substrates modified by plasma deposited surface coating from methyl oxazoline, octadiene and acrylic acid chemistries. (A) aluminum, (B) aluminum carbon ratio, (C) oxygen carbon ratio and (D) nitrogen carbon ratio.

The surface chemical composition of the coatings deposited by plasma polymerization on NAA substrates was characterized by XPS. The unmodified NAA substrates had 35 atomic percent of aluminum (**Figure 3A**). After deposition of a 5 nm thin plasma

1
2
3 polymer coating of Meoxpp, ODpp and ACpp, the atomic percentage of aluminum
4
5
6
7 decreased by half, which indicates that the coatings were successfully deposited on
8
9
10 the NAA substrates. Furthermore, the aluminum to carbon (Al/C) and oxygen to carbon
11
12
13 (O/C) ratios decreased by one fourth in case of Meoxpp and ODpp coated nanoporous
14
15
16 and NAA substrates as compared to their uncoated counterparts (**Figures 3B and C**).
17
18
19 This is due to the high concentration of carbon present in the molecules of these
20
21
22 monomers. Whilst Al/C ratio decreased by half and O/C ratio remained the same in
23
24
25 ACpp overcoated NAA substrates compared to their uncoated analogs (**Figures 3B**
26
27
28 **and C**). This is due to high concentration of oxygen present in the structure of acrylic
29
30
31 acid. Moreover, alumina membrane has a top oxide layer which leads to high O/C ratio
32
33
34 in case of uncoated alumina surface. The nitrogen by carbon (N/C) ratio is presented
35
36
37 in **Figure 3D**. Nitrogen was detected only in case of Meoxpp and not in case of ODpp
38
39
40 and ACpp coated surfaces constant with the chemical structure of the precursors.
41
42
43
44
45
46
47
48
49 Inflammatory responses (pro-inflammatory and anti-inflammatory) on different
50
51
52
53 chemistry and nanoporosity
54
55
56
57
58
59
60

Macrophages play a central role in the host response to implanted biomaterials. These cells have the capability to polarize into M1 (inflammatory) and M2 (wound healing) phenotypes, which further generate an array of pro-inflammatory and anti-inflammatory cytokines, respectively. In this study, inflammatory responses to surface chemistry and nanoporosity (individually and in combination) was assessed in culture of macrophage dTHP-1 cells, obtained from differentiated THP-1 cell line. The results are presented in Figures 4 and 5.



1
2
3
4 **Figure 4:** Expression of pro-inflammatory cytokines (TNF α , IL-6, and IL-1 β) from
5
6
7 macrophages stimulated by LPS (1 μ g/ml) to nanoporous alumina surfaces with
8
9
10 different pore size and surface chemistry, as determined using Legendplex ELISAs.
11
12
13
14 TNF α , IL-6, and IL-1 β expression on nanoporous alumina modified with methyl
15
16
17 oxazoline (Meox), octadiene (OD) and acrylic acid AC (A, D & G)), uncoated alumina
18
19
20 with different pore sizes 30 nm, 65 nm and 200 nm (B, E & H), and from NAA modified
21
22
23
24 with Meoxpp, ODpp and ACpp (C, F&I). * = p<0.05, ** = p<0.01 and *** = p<0.001
25
26
27

28
29 An overall reduction in cytokine expression levels was observed upon addition of a
30
31
32 combination of nanoporosity and chemistry compared to the uncoated aluminium
33
34
35 membranes (**Figure 4**). In the case of Meoxpp (** = p<0.01) and ACpp (* = p<0.05)
36
37
38 coated surfaces, a significant reduction in the concentration of the cytokine tumor
39
40
41
42 necrosis factor alpha (TNF α) was observed, while only a moderate reduction in
43
44
45 concentration of interleukin-6 (IL-6) and interleukin-1 β (IL-1 β) was observed as
46
47
48
49 compared to aluminum control surfaces (**Figures 4 A, D and G**). Upon addition of
50
51
52
53 nanoporosity, a significant increase in the concentration of all three cytokines (TNF α ,
54
55
56 IL-6 and IL-1 β) was observed on 30 nm surfaces, while the cytokine expression
57
58
59
60

1
2
3 decreased significantly on surfaces with larger pore size (i.e. 65 nm and 200 nm)
4
5
6
7 **(Figures 4 B, E and H).**
8
9

10 The combination of nanoporosity and surface chemistry led to an overall significant
11
12 decrease in the concentration of proinflammatory TNF α and IL-6 cytokines (compared
13
14 to the control aluminum surfaces **(Figures 4 C, F and I)**. Though an overall decrease
15
16 in cytokines level was observed, the chemistries in combination with large pore size
17
18 (200 nm) showed a more prominent decrease in the cytokine concentration compared
19
20 to same chemistries on smaller pore sizes (i.e. 65 nm and 30 nm). Furthermore,
21
22 nanoporous surfaces overcoated with Meoxpp and ACpp showed significant reduction
23
24 in expression of all three pro-inflammatory cytokines compared to planar surfaces as
25
26 well as to nanoporous surfaces with ODpp overcoating. The most significant reduction
27
28 in the inflammatory cytokines was observed in case of Meoxpp and ACpp overcoated
29
30 200 nm (large pore diameter) surface. In case of nanoporous surfaces overcoated with
31
32 ODpp chemistry, the expression of TNF α and IL-6 cytokines decreased significantly
33
34 with the increase in pore size while an overall increase in the concentration of IL-1 β
35
36 was observed on these surfaces compared to planar alumina surfaces.
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Table 1. Heat map summarizing the results obtained from ANOVA analysis performed to differentiate the individual effect of nanotopography and chemistry in the combination of the two, in case of pro-inflammatory cytokines expression.

Biomarkers	Nanotopography	Chemistry	Combination
TNF α	84%	2%	14%
IL-6	79%	7%	14%
IL1 β	32%	66%	2%

Our experimental data shows (Figure 4) that the combination of nanotopography and chemistry has a synergistic effect on pro-inflammatory cytokine expression. However, to determine, the weighted impact of individual surface parameters in case of combination of t nanotopography and chemistry, a two way ANOVA was performed. The results presented in Table 1 were plotted by using the F0 values presented in supplementary Table S1. The ANOVA analysis demonstrates that in the case of TNF α and IL-6 nanotopography plays a much more significant role (83.9 % and 78.9%, respectively) compared to surface chemistry (1.9% and 6.7% respectively). Whereas, in case of IL-1 β , both nanotopography (32.2%) and chemistry (66%) appear to be

important, however, surface chemistry has more prominent effect than nanotopography.

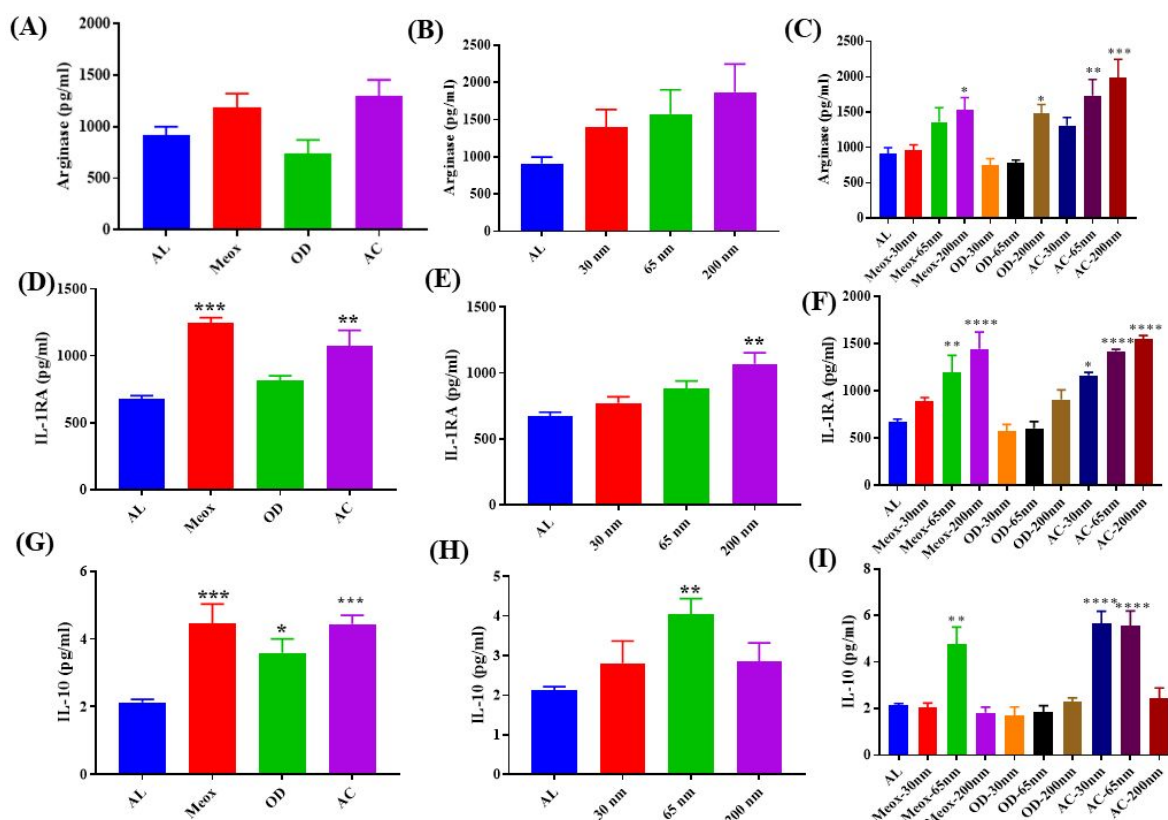


Figure 5: Expression of anti-inflammatory cytokines (Arginase, IL-1RA and IL-10) from macrophages stimulated by LPS (1 µg/ml) to nanoporous alumina surfaces with different pore size and surface chemistry, as determined using Legendplex ELISAs. Cytokines Arginase, IL-1RA and IL-10 on nanoporous alumina modified with oxazoline (Meox), octadiene (OD) and acrylic acid (AC) plasma polymer (A, D & G), alumina

1
2
3 with different pore sizes 30 nm, 65 nm and 200 nm (B, E &H), and from NAA modified
4
5
6
7 with Meox, OD and AC (C, F&I). * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$
8
9

10
11 An overall increment in anti-inflammatory cytokine concentration upon combination of
12
13
14 nanoporosity and chemistry for all surface chemical modifications was observed
15
16
17
18 **(Figure 5)**. Meoxpp and ACpp coated surfaces displayed a significant increase in the
19
20
21 concentrations of the cytokines, interleukin 1 receptor antagonist (IL-1RA) and
22
23
24 interleukin-10 (IL-10), and only a moderate increment in concentration of arginase
25
26
27 compared to aluminum control surfaces **(Figure 5 A, D and G)**. Upon addition of
28
29
30
31 nanoporosity, the concentration of IL-1RA increased significantly on surfaces with
32
33
34 larger nanopores (200 nm) compared to counterpart surfaces with smaller nanopores
35
36
37
38 (30 nm and 65 nm). While an increase in the concentration of IL-10 was more
39
40
41
42 significant on 65 nm compared to 30 nm and 200 nm surfaces, and only a moderate
43
44
45 increase in concentration of arginase (as compared to aluminum control surfaces) was
46
47
48
49 observed **(Figures 5 B, E and H)**.
50
51

52
53 The combination of different nanoporosity with these chemistries led to an overall
54
55
56 significant increase in the concentration of the anti-inflammatory cytokines (arginase,
57
58
59 IL1RA and IL-10) compared to the control aluminum surfaces **(Figures 5 C, F and I)**.
60

1
2
3
4 Although, an overall increase in cytokines level was observed, the chemistries in
5
6
7 combination with large pore size showed a more prominent increase in the cytokine
8
9
10 concentration compared to chemistries on smaller pore size. Furthermore, anti-
11
12
13 inflammatory cytokines increased to a much greater extent on nanoporous surfaces
14
15
16 overcoated with Meoxpp and ACpp overcoated surfaces compared to ODpp
17
18
19 overcoated nanoporous surfaces. The most significant increase in the expression of
20
21
22 Arginase and IL1RA was observed in case of Meoxpp and ODpp overcoated 65 nm
23
24
25 and 200 nm surfaces. While the most significant increase in the concentration IL-10
26
27
28 was observed in case of ACpp overcoated 30 nm and 65 nm surfaces.
29
30
31
32
33
34
35

36 Table 2. Heat map summarizing the obtained results from ANOVA analysis performed
37
38
39 to differentiate the individual effect of nanotopography and chemistry in the
40
41
42 combination of the two, in case of anti-inflammatory cytokines.
43
44
45
46

Biomarkers	Nanotopography	Chemistry	Combination
Arginase	48%	49%	3%
IL1RA	27%	72%	1%
IL10	27%	55%	18%

1
2
3
4 Two-way ANOVA analysis was performed to determine the weighted contribution of
5
6
7 individual surface properties. The results in Table 2 were obtained by using the F0
8
9
10 values from supplementary Table S2. Table 2, clearly demonstrates that in the case
11
12
13 of Arginase, IL1RA and IL10 both chemistry and nanotopography plays a significant
14
15
16 role. However, in case of IL1RA, chemistry is three times more significant than
17
18
19 nanotopography while in case of IL10, chemistry is only twice as significant as
20
21
22 nanotopography while in case of IL10, chemistry is only twice as significant as
23
24
25 nanotopography.
26
27

28 29 DISCUSSION

30
31
32 It is well established that different surface features play a critical role in modulating
33
34
35 inflammatory responses ¹³⁻¹⁷. However, the effect of these surface features on
36
37
38 macrophages are still not well known. Macrophages become activated into 'M1' and
39
40
41 'M2' phenotype and expresses (pro and anti) inflammatory cytokines on its interaction
42
43
44 with the biomaterial. While an initial pro inflammatory response to biomaterials
45
46
47 generated by M1 macrophages is desired, its prolonged expression results in chronic
48
49
50 inflammatory events followed by the formation of FBGC and failure of biomaterial
51
52
53 implant. In addition, 'M2' phenotype expresses anti-inflammatory cytokines which
54
55
56
57
58
59
60

1
2
3 promotes tissue remodeling and aids in vascularization of regenerative biomaterials,
4
5
6
7 inhibiting fibrous capsule formation. This suggests that controlling the fate of
8
9
10
11 macrophage polarization is beneficial in retaining the integrity and normal functioning
12
13
14 of the biomaterial implant. Therefore, understanding macrophage polarization through
15
16
17 modulation of surface features has critical implications on the design and engineering
18
19
20
21 of implantable biomaterials. This study reveals that a combination of surface
22
23
24 nanoporosity with tailored surface chemistry can be readily used to modulate
25
26
27
28 macrophage polarization by modulating the secretion of pro-inflammatory and anti-
29
30
31 inflammatory cytokines.
32

33
34
35 NAA substrates were fabricated by two-step anodization to tune the geometric
36
37
38 features of nanopores with precision³⁵⁻³⁸. A thin layer of Meoxpp, ODpp and ACpp (~5
39
40
41 nm) was deposited onto these nanoporous surfaces to generate desired uniform
42
43
44
45 surface chemistries to further modulate macrophage responses. Using this approach,
46
47
48
49 we were able to generate 15 independent types of surfaces with unique combinations
50
51
52
53 of nanoporosity and chemistry. FEG-SEM and XPS analysis were used to establish
54
55
56
57 the geometric and chemical features of the nanoporous substrates.
58
59
60

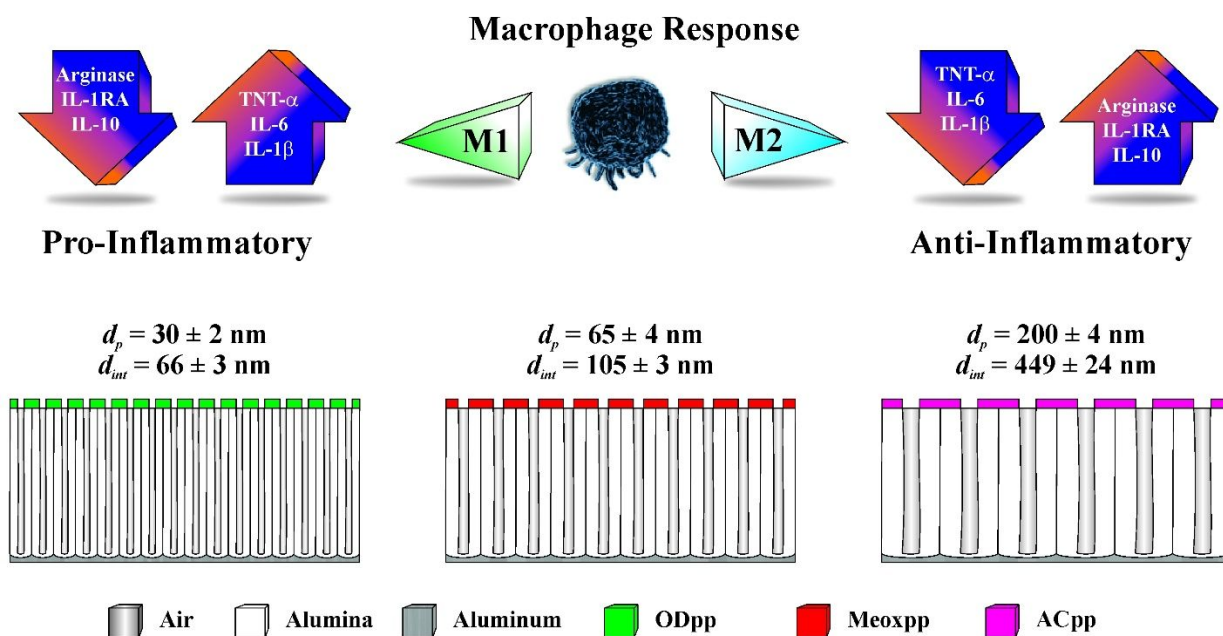
1
2
3
4 Our data demonstrates that the expression of pro-inflammatory cytokines decreased
5
6
7 while anti-inflammatory cytokines increased on Meoxpp and ACpp coated surfaces,
8
9
10 and the effect was boosted by combining these chemistries with increasing surface
11
12
13 nanoporosity (30<65<200 nm). This is consistent with previous studies showing that
14
15
16 hydrophilic -COOH surfaces can reduce inflammatory responses and fibrotic
17
18
19 encapsulation⁴⁸⁻⁴⁹. Our previous studies also demonstrated that the combination of
20
21
22 nanotopography (nanohills) with the -COOH surfaces reduced the level of expression
23
24
25 of proinflammatory cytokines ¹⁴ and fibrotic capsule thickness¹⁶ while increasing the
26
27
28 expression of collagens⁴⁷. Furthermore, the results presented in this paper indicate
29
30
31 that the expression of cytokines can be modulated to a greater extent by using
32
33
34 nanoporous surfaces with same chemistry compared to gold nanoparticles
35
36
37 nanotopography surfaces with the same chemistry¹⁴. It is noteworthy that the cell
38
39
40 numbers were same across all 15 substrates (**Figure S3 and S4**). Therefore, the
41
42
43 decrease in pro-inflammatory signals or the increase in anti-inflammatory signals were
44
45
46 not affected by adherent cells.
47
48
49
50
51
52
53

54
55
56 Furthermore, the levels of TNF α decreased significantly on Meoxpp and ACpp coated
57
58
59 surfaces as well as on nanoporous surfaces with greater pore size compared to
60

1
2
3 uncoated alumina membranes. But the reduction in IL-6 and IL-1 β expression was
4
5
6
7 only observed upon combination of nanoporosity with surface chemistry. Additionally,
8
9
10 IL-1RA and IL-10 increased significantly on Meoxpp and ACpp surfaces and remained
11
12
13 unchanged on different nanoporous surfaces compared to uncoated alumina
14
15
16 membranes. However, concentration of arginase only increased when a combination
17
18
19 of nanoporosity with chemistry was utilized. Interestingly, hydrophobic ODpp surfaces
20
21
22 showed no change in inflammatory responses (pro and anti). Also, expression of TNF α
23
24
25 decreased while IL-1RA increased, but there was no change observed in the
26
27
28 concentration of IL-6, IL-1 β , Arginase and IL-10 on surfaces with greater nanoporosity
29
30
31
32
33
34
35 (30<65<200 nm).
36
37
38

39 ODpp and nanoporous surfaces alone have been known to enhance inflammatory
40
41
42 responses and fibrotic encapsulation around biomaterials⁴⁹⁻⁵². On the contrary, our
43
44
45 data indicates a significant reduction in the expression of TNF α and IL-6, and an
46
47
48 increase in the expression of arginase when ODpp and large surface pores are used
49
50
51
52
53 together. This suggests that surface nanoporosity or surface chemistry cannot be used
54
55
56 alone as a tool to modulate immune responses. A possible explanation for
57
58
59 nanoporosity mediated macrophage polarization could be nanotopography induced
60

orientation and conformational changes of adhered proteins⁵³⁻⁵⁴, exposing unknown cell-surface receptors, causing unprecedented cellular behavior¹⁷.



Schematic 1. Schematic representation of macrophage polarization (M1 and M2 phenotypes) on chemically and nanotopographically modified surfaces.

One of the biggest problems with medical devices is fibrosis or fibrous encapsulation⁵⁵⁻⁵⁸. Several strategies involving addition of growth factors⁵⁹⁻⁶², surface chemical modifications^{46, 63-65} or the addition of surface nanotopography^{13, 66-68} have been explored to address these problems. The data presented here suggest that the combinatorial effect of surface nanoporosity and surface chemistry can be used to control macrophage differentiation by modulating pro-inflammatory and anti-

1
2
3
4 inflammatory cytokines. For example, large pores and ACpp based outermost surface
5
6
7 chemistry was shown to reduce expression of proinflammatory cytokines (**Figure 4**)
8
9
10 and increase the production of arginase (**Figure 5**) suggesting differentiation to M2
11
12
13 type macrophages which contribute to improved healing. This has been further
14
15
16 illustrated in **schematic 1**. To the best of our knowledge, this is the first comprehensive
17
18
19 study, demonstrating that macrophage differentiation can be controlled by utilizing the
20
21
22 right combination of surface nanoporosity and chemistry. As pro-inflammatory
23
24
25 cytokines decreased, and anti-inflammatory cytokines increased on -COOH surfaces
26
27
28 with large nanopores. Such surfaces can be utilized to fabricate biomaterials that can
29
30
31 tune immune responses to enhance implantation site healing and can be used to
32
33
34 establish a base for the future rational design of biomaterial implants.
35
36
37
38
39
40
41
42

43 CONCLUSION

44
45
46 In this study, the role of surface nanoporosity and chemistry in controlling
47
48
49 macrophages polarization into 'M1' and 'M2' phenotypes was assessed. Controlled
50
51
52 surface nanotopography was generated by utilizing three different sizes of surface
53
54
55 pores (30, 65 and 200 nm). Whereas, desired outermost surface chemistry on NAA
56
57
58
59
60

1
2
3 substrates was generated by coating 5 nm plasma polymer layer obtained from
4
5
6
7 different monomers such as methyl oxazoline, 1, 7 octadiene and acrylic acid. This
8
9
10 model system enabled the evaluation of the interplay between surface
11
12
13 nanotopography and chemistry. We have shown that the concentration of pro-
14
15
16
17 inflammatory cytokines (TNF α , IL-6 and IL-1 β) decreased significantly on nanoporous
18
19
20
21 surfaces featuring large nanopores and having Meoxpp and ACpp surface coatings
22
23
24 compared to surfaces with smaller pore sizes and methyl group rich chemistry (OD).
25
26
27
28 Furthermore, the concentration of anti-inflammatory cytokines (Arginase, IL-1RA and
29
30
31 IL-10) increased significantly on large nanoporous surfaces with Meoxpp and ACpp
32
33
34 coatings. Our data suggests that the macrophage differentiation can be controlled by
35
36
37
38 selecting desired combinations of surface nanoporosity and chemistry. The knowledge
39
40
41
42 obtained from this study provides cues that could aid in tuning foreign body responses
43
44
45 and will eventually facilitate the rational design of biomaterial implants and constructs.
46
47
48

49 ASSOCIATED CONTENT

50
51
52
53 Supporting Information
54
55
56
57
58
59
60

1
2
3
4 The Supporting Information is available free of charge on the ACS Publications
5
6
7 website at DOI:
8
9

10
11 Zeta Potential Measure (Figure S1) and water contact angle (Figure S2) of chemically
12
13
14 modified surfaces. Macrophage cell count (Figure S3 and S4) on chemically and
15
16
17
18 nanotopographically modified surfaces.
19

20 21 22 AUTHOR INFORMATION 23

24
25 *Corresponding Authors:
26

27
28 Dr Akash Bachhuka
29

30
31
32 ARC Center of Excellence for Nanoscale BioPhotonics, Institute for Photonics and
33
34
35 Advanced Sensing, Faculty of Science, The University of Adelaide, South Australia,
36
37
38 Australia, 5005.
39

40
41
42 Phone: (61) 424446844
43

44
45
46 Email: akash.bachhuka@adelaide.edu.au
47
48

49
50 Professor Krasimir Vasilev
51
52
53
54
55
56
57
58
59
60

1
2
3
4 Division of Information Technology, Engineering and the Environment, Future
5
6
7 Industries Institute, University of South Australia, Mawson Lakes Campus, South
8
9
10 Australia, Australia, 5095.

11
12
13
14 Phone: (61) 8 8302 5697, Fax: (61) 8 8302 5689

15
16
17 Email: krasimir.vasilev@unisa.edu.au

18 19 20 21 AUTHOR CONTRIBUTIONS

22
23
24
25 The manuscript was written through contributions of all authors. All authors have given
26
27
28 approval to the final version of the manuscript.

29 30 31 32 ACKNOWLEDGMENT

33
34
35
36 KV thank to ARC grant ARC DP15104212, NHMRC for Fellowship APP1122825 and
37
38
39 the Humboldt Foundation for the Humboldt Fellowship for Experienced Researchers.
40
41
42
43 AB, AS and HEH thank to ARC grant ARC CE140100003.

44 45 46 47 48 49 50 REFERENCES

- 1
2
3
4 1. Vroman L, Adams A, Fischer G, Munoz P. Interaction of high molecular weight
5
6
7 kininogen, factor XII, and fibrinogen in plasma at interfaces. *Blood*. 1980; 55 (1): 156-
8
9
10 159.
11
12
- 13
14 2. Hirsh SL, McKenzie DR, Nosworthy NJ, Denman JA, Sezerman OU, Bilek MMM.
15
16
17 The Vroman effect: Competitive protein exchange with dynamic multilayer protein
18
19
20 aggregates. *Colloids Surf. B. Biointerfaces*. 2013; 103: 395-404.
21
22
- 23
24 3. Nimeri G, Öhman L, Elwing H, Wetterö J, Bengtsson T. The influence of plasma
25
26
27 proteins and platelets on oxygen radical production and F-actin distribution in
28
29
30 neutrophils adhering to polymer surfaces. *Biomaterials*. 2002; 23 (8): 1785-1795.
31
32
- 33
34 4. Nimeri G, Majeed M, Elwing H, Öhman L, Wetterö J, Bengtsson T. Oxygen radical
35
36
37 production in neutrophils interacting with platelets and surface-immobilized plasma
38
39
40 proteins: Role of tyrosine phosphorylation. *J. Biomed. Mater. Res. A*. 2003; 67A (2):
41
42
43 439-447.
44
45
46
- 47
48 5. Wetterö J, Bengtsson T, Tengvall P. Complement activation on immunoglobulin G-
49
50
51 coated hydrophobic surfaces enhances the release of oxygen radicals from
52
53
54 neutrophils through an actin-dependent mechanism. *J. Biomed. Mater. Res*. 2000; 51
55
56
57 (4): 742-751.
58
59
60

- 1
2
3
4 6. Lynn AD, Kyriakides TR, Bryant SJ. Characterization of the in vitro macrophage
5
6
7 response and in vivo host response to poly(ethylene glycol)-based hydrogels. J.
8
9
10 Biomed. Mater. Res. A. 2010; 93A (3): 941-953.
11
12
- 13
14 7. Zhao Q, Topham N, Anderson JM, Hiltner A, Lodoen G, Payet CR. Foreign-body
15
16
17 giant cells and polyurethane biostability: In vivo correlation of cell adhesion and
18
19
20 surface cracking. J. Biomed. Mater. Res. 1991; 25 (2): 177-183.
21
22
- 23
24 8. Christo SN, Diener KR, Bachhuka A, Vasilev K, Hayball JD. Innate Immunity and
25
26
27 Biomaterials at the Nexus: Friends or Foes. BioMed Research International. 2015;
28
29
30 2015:1155-78.
31
32
- 33
34 9. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and
35
36
37 polarization. Front. Biosci. 2008; 13 (1): 453-61.
38
39
- 40
41 10. Mosser DM. The many faces of macrophage activation. J. Leukoc. Biol. 2003; 73
42
43
44 (2): 209-212.
45
46
47
- 48
49 11. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine
50
51
52 system in diverse forms of macrophage activation and polarization. Trends Immunol.
53
54
55 2004; 25 (12): 677-686.
56
57
58
59
60

1
2
3
4 12. Martinez FO, Helming L, Gordon S. Alternative Activation of Macrophages: An
5
6
7 Immunologic Functional Perspective. *Annu. Rev. Immunol.* 2009; 27 (1): 451-483.
8
9

10
11 13. Chen Z, Bachhuka A, Han S, Wei F, Lu S, Visalakshan RM, Vasilev K, Xiao Y.
12
13
14 Tuning chemistry and topography of nanoengineered surfaces to manipulate immune
15
16
17 response for bone regeneration applications. *ACS Nano.* 2017; 11 (5): 4494-4506.
18
19

20
21 14. Christo SN, Bachhuka A, Diener KR, Mierczynska A, Hayball JD, Vasilev K. The
22
23
24 role of surface nanotopography and chemistry on primary neutrophil and macrophage
25
26
27 cellular responses. *Advanced healthcare materials.* 2016; 5 (8): 956-965.
28
29

30
31 15. Christo S, Bachhuka A, Diener KR, Vasilev K, Hayball JD. The contribution of
32
33
34 inflammasome components on macrophage response to surface nanotopography and
35
36
37 chemistry. *Sci. Rep.* 2016; 18 (6): 26207.
38
39

40
41 16. Bachhuka A, Christo SN, Cavallaro A, Diener KR, Mierczynska A, Smith LE,
42
43
44 Marian R, Manavis J, Hayball JD, Vasilev K. Hybrid core/shell microparticles and their
45
46
47 use for understanding biological processes. *J. Colloid Interface Sci.* 2015; 457: 9-17.
48
49

50
51 17. Visalakshan RM, Cavallaro AA, MacGregor MN, Lawrence EP, Koynov K, Hayball
52
53
54 JD, Vasilev K. Nanotopography-Induced Unfolding of Fibrinogen Modulates Leukocyte
55
56
57 Binding and Activation. *Adv. Funct. Mater.* 2019; 29 (14): 1807453.
58
59
60

1
2
3
4 18. Santos A, Aw MS, Bariana M, Kumeria T, Wang Y, Losic D. Drug-releasing
5
6
7 implants: current progress, challenges and perspectives. Journal of Materials
8
9
10 Chemistry B. 2014; 2 (37): 6157-6182.

11
12
13
14 19. Losic D, Simovic S. Self-ordered nanopore and nanotube platforms for drug
15
16
17 delivery applications. Expert opinion on drug delivery. 2019; 2 (37): 6157-6182.

18
19
20
21 20. La Flamme KE, Popat KC, Leoni L, Markiewicz E, La Tempa TJ, Roman BB,
22
23
24 Grimes CA, Desai TA. Biocompatibility of nanoporous alumina membranes for
25
26
27 immunoisolation. Biomaterials 2007; 28 (16): 2638-2645.

28
29
30
31 21. Popat KC, Swan EEL, Mukhatyar V, Chatvanichkul KI, Mor GK, Grimes CA, Desai
32
33
34 TA. Influence of nanoporous alumina membranes on long-term osteoblast response.
35
36
37 Biomaterials 2005; 26 (22): 4516-4522.

38
39
40
41 22. Swan EEL, Popat KC, Desai TA. Peptide-immobilized nanoporous alumina
42
43
44 membranes for enhanced osteoblast adhesion. Biomaterials. 2005; 26 (14): 1969-
45
46
47 1976.

48
49
50
51 23. Popat KC, Chatvanichkul KI, Barnes GL, Latempa Jr TJ, Grimes CA, Desai TA.
52
53
54 Osteogenic differentiation of marrow stromal cells cultured on nanoporous alumina
55
56
57 surfaces. J. Biomed. Mater. Res. A. 2007; 80 (4): 955-964.
58
59
60

1
2
3
4 24. Karlsson M, Pålsgård E, Wilshaw PR, Di Silvio L. Initial in vitro interaction of
5
6
7 osteoblasts with nano-porous alumina. *Biomaterials*. 2003; 24 (18): 3039-3046.
8
9

10
11 25. Michelmore A, Martinek P, Sah V, Short RD, Vasilev K. Surface Morphology in the
12
13
14 Early Stages of Plasma Polymer Film Growth from Amine-Containing Monomers.
15
16
17 *Plasma Processes and Polymers*. 2011; 8 (5): 367-372.
18
19

20
21 26. Hernandez-Lopez J, Bauer R, Chang WS, Glasser G, Grebel-Koehler D, Klapper
22
23
24 M, Kreiter M, Leclair J, Majoral JP, Mittler S. Functional polymers as nanoscopic
25
26
27 building blocks. *Materials Science and Engineering: C* 2003; 23 (1-2): 267-274.
28
29

30
31 27. Rinsch CL, Chen XL, Panchalingam V, Eberhart RC, Wang JH, Timmons RB.
32
33
34 Pulsed radio frequency plasma polymerization of allyl alcohol: Controlled deposition
35
36
37 of surface hydroxyl groups. *Langmuir*. 1996; 12 (12): 2995-3002.
38
39

40
41
42 28. Michelmore A, Steele DA, Whittle JD, Bradley JW, Short RD. Nanoscale deposition
43
44
45 of chemically functionalised films via plasma polymerisation. *Rsc Advances*. 2013; 3
46
47
48 (33): 13540-13557.
49

50
51
52 29. Coad BR, Scholz T, Vasilev K, Hayball JD, Short RD, Griesser HJ. Functionality
53
54
55 of proteins bound to plasma polymer surfaces. *ACS applied materials & interfaces*.
56
57
58 2012; 4 (5): 2455-2463.
59
60

- 1
2
3
4 30. Goreham RV, Mierczynska A, Pierce M, Short RD, Taheri S, Bachhuka A,
5
6
7 Cavallaro A, Smith LE, Vasilev K. A substrate independent approach for generation of
8
9
10 surface gradients. *Thin Solid Films*. 2013; 528: 106-110.
11
12
13
14 31. Vasilev K, Poulter N, Martinek P, Griesser HJ. Controlled release of levofloxacin
15
16
17 sandwiched between two plasma polymerized layers on a solid carrier. *ACS Applied*
18
19
20 *Materials & Interfaces* 2011; 3 (12): 4831-4836.
21
22
23
24 32. Wahono SK, Cavallaro A, Vasilev K, Mierczynska A. Plasma polymer facilitated
25
26
27 magnetic technology for removal of oils from contaminated waters. *Environ. Pollut.*
28
29
30
31 2018; 240: 725-732.
32
33
34
35 33. Mierczynska-Vasilev A, Boyer P, Vasilev K, Smith PA. A novel technology for the
36
37
38 rapid, selective, magnetic removal of pathogenesis-related proteins from wines. *Food*
39
40
41 *Chem*. 2017; 232: 508-514.
42
43
44
45 34. Cavallaro A, Vasilev K. Controlled and sustained release of pharmaceuticals via
46
47
48 single step solvent-free encapsulation. *Chem. Commun*. 2015; 51 (10): 1838-1841.
49
50
51
52 35. Masuda H, Fukuda K. Ordered metal nanohole arrays made by a two-step
53
54
55 replication of honeycomb structures of anodic alumina. *Science*. 1995; 268 (5216):
56
57
58 1466-1468.
59
60

- 1
2
3
4 36. Nielsch K, Choi J, Schwirn K, Wehrspohn RB, Gösele U. Self-ordering regimes of
5
6
7 porous alumina: the 10 porosity rule. *Nano Lett.* 2002; 2 (7): 677-680.
8
9
- 10 37. Lee W, Park S-J. Porous anodic aluminum oxide: anodization and templated
11
12
13 synthesis of functional nanostructures. *Chem. Rev.* 2014; 114 (15): 7487-7556.
14
15
16
- 17 38. Santos A, Kumeria T, Losic D. Nanoporous anodic aluminum oxide for chemical
18
19
20 sensing and biosensors. *TrAC, Trends Anal. Chem.* 2013; 44: 25-38.
21
22
23
- 24 39. Chanput W, Mes J, Vreeburg RAM, Savelkoul HFJ, Wichers HJ. Transcription
25
26
27 profiles of LPS-stimulated THP-1 monocytes and macrophages: a tool to study
28
29
30 inflammation modulating effects of food-derived compounds. *Food & Function.* 2010;
31
32
33
34 1 (3): 254-261.
35
36
37
- 38 40. Chanput W, Mes JJ, Savelkoul HFJ, Wichers HJ. Characterization of polarized
39
40
41 THP-1 macrophages and polarizing ability of LPS and food compounds. *Food &*
42
43
44
45 *Function.* 2013; 4 (2): 266-276.
46
47
48
- 49 41. Chanput W, Mes JJ, Wichers HJ. THP-1 cell line: An in vitro cell model for immune
50
51
52 modulation approach. *International Immunopharmacology.* 2014; 23 (1): 37-45.
53
54
55
- 56 42. Kessel A, Ben-Tal N. Introduction to proteins: structure, function, and motion. CRC
57
58
59 Press. 2010.
60

1
2
3
4 43. Zheng K, Kapp M, Boccaccini AR. Protein interactions with bioactive glass
5
6
7 surfaces: A review. *Applied Materials Today*. 2019; 15: 350-371.
8
9

10
11 44. Visalakshan RM, MacGregor MN, Cavallaro AA, Sasidharan S, Bachhuka A,
12
13
14 Vasilev AM, Hayball JD, Vasilev K. Creating Nano-engineered Biomaterials with Well-
15
16
17 Defined Surface Descriptors. *ACS Applied Nano Materials*. 2018; 1 (6): 2796-2807.
18
19

20
21 45. Mierczynska-Vasilev A, Mierczynski P, Maniukiewicz W, Visalakshan RM, Vasilev
22
23
24 K, Smith PA. Magnetic separation technology: Functional group efficiency in the
25
26
27 removal of haze-forming proteins from wines. *Food Chemistry*. 2019; 275: 154-160.
28
29

30
31 46. Bachhuka A, Hayball J, Smith LE, Vasilev K. Effect of surface chemical
32
33
34 functionalities on collagen deposition by primary human dermal fibroblasts. *ACS*
35
36
37 *Applied Materials & Interfaces*. 2015; 7 (42): 23767-23775.
38
39

40
41 47. Bachhuka A, Hayball JD, Smith LE, Vasilev K. The interplay between surface
42
43
44 nanotopography and chemistry modulates collagen I and III deposition by human
45
46
47 dermal fibroblasts. *ACS Applied Materials & Interfaces*. 2017; 9 (7): 5874-5884.
48
49

50
51 48. Kamath S, Bhattacharyya D, Padukudru C, Timmons RB, Tang L. Surface
52
53
54 chemistry influences implant-mediated host tissue responses. *J. Biomed. Mater. Res.*
55
56
57
58
59 A. 2008; 86 (3): 617-626.
60

1
2
3
4 49. Barbosa JN, Madureira P, Barbosa MA, Aguas AP. The influence of functional
5
6
7 groups of self-assembled monolayers on fibrous capsule formation and cell
8
9
10 recruitment. *J. Biomed. Mater. Res. A.* 2006; 76 (4): 737-743.

11
12
13
14 50. Damanik FFR, Rothuizen TC, van Blitterswijk C, Rotmans JI, Moroni L. Towards
15
16
17 an in vitro model mimicking the foreign body response: tailoring the surface properties
18
19
20 of biomaterials to modulate extra-cellular matrix. *Sci. Rep.* 2014; 4: 6325.

21
22
23
24 51. Ferraz N, Hong J, Santin M, Karlsson Ott M. Nanoporosity of alumina surfaces
25
26
27 induces different patterns of activation in adhering monocytes/macrophages.
28
29
30 *International journal of biomaterials.* 2010; 2010: 1-8.

31
32
33
34 52. Pujari S, Hoess A, Shen J, Thormann A, Heilmann A, Tang L, Karlsson-Ott M.
35
36
37 Effects of nanoporous alumina on inflammatory cell response. *J. Biomed. Mater. Res.*
38
39
40
41
42 A. 2014; 102 (11): 3773-3780.

43
44
45 53. Roach P, Eglin D, Rohde K, Perry CC. Modern biomaterials: a review—bulk
46
47
48 properties and implications of surface modifications. *J. Mater. Sci. Mater. Med.* 2007;
49
50
51
52 18 (7): 1263-1277.

53
54
55 54. Wilson CJ, Clegg RE, Leavesley DI, Percy MJ. Mediation of biomaterial–cell
56
57
58 interactions by adsorbed proteins: a review. *Tissue Eng.* 2005; 11 (1-2): 1-18.
59
60

- 1
2
3
4 55. Chen CZ, Raghunath M. Focus on collagen: in vitro systems to study fibrogenesis
5
6
7 and antifibrosis _ state of the art. *Fibrogenesis & Tissue Repair*. 2009; 2 (1): 7.
8
9
10 56. Holt DJ, Grainger DW. Multinucleated giant cells from fibroblast cultures.
11
12
13 *Biomaterials*. 2011; 32 (16): 3977-3987.
14
15
16
17 57. Kamath S, Bhattacharyya D, Padukudru C, Timmons RB, Tang L. Surface
18
19
20 chemistry influences implant-mediated host tissue responses. *J. Biomed. Mater. Res.*
21
22
23
24 A. 2008; 86A (3): 617-626.
25
26
27
28 58. Eaton JW, Tang L. Inflammatory Responses to Biomaterials. *Am. J. Clin. Pathol.*
29
30
31 1995; 103 (4): 466-471.
32
33
34
35 59. Moreira M, Fagundes DJ, Simões MdJ, Taha MO, Perez LMN, Bazotte RB. The
36
37
38 effect of liposome-delivered prednisolone on collagen density, myofibroblasts, and
39
40
41 fibrous capsule thickness around silicone breast implants in rats. *Wound Repair*
42
43
44
45 *Regen*. 2010; 18 (4): 417-425.
46
47
48
49 60. Avula M, Jones D, Rao AN, McClain D, McGill LD, Grainger DW, Solzbacher F.
50
51
52 Local release of masitinib alters in vivo implantable continuous glucose sensor
53
54
55 performance. *Biosens. Bioelectron*. 2016; 77: 149-156.
56
57
58
59
60

1
2
3
4 61. Kastellorizios M, Papadimitrakopoulos F, Burgess DJ. Multiple tissue response
5
6
7 modifiers to promote angiogenesis and prevent the foreign body reaction around
8
9
10 subcutaneous implants. *J. Control. Release.* 2015; 214: 103-111.

11
12
13
14 62. Shin YN, Kim BS, Ahn HH, Lee JH, Kim KS, Lee JY, Kim MS, Khang G, Lee HB.
15
16
17 Adhesion comparison of human bone marrow stem cells on a gradient wettable
18
19
20 surface prepared by corona treatment. *Appl. Surf. Sci.* 2008; 255 (2): 293-296.

21
22
23
24 63. Tang L, Thevenot P, Hu W. Surface chemistry influences implant biocompatibility.
25
26
27
28 *Curr. Top. Med. Chem.* 2008; 8 (4): 270-280.

29
30
31 64. Liu X, Feng Q, Bachhuka A, Vasilev K. Surface modification by allylamine plasma
32
33
34 polymerization promotes osteogenic differentiation of human adipose-derived stem
35
36
37 cells. *ACS Applied Materials & Interfaces.* 2014; 6 (12): 9733-9741.

38
39
40
41 65. Liu X, Feng Q, Bachhuka A, Vasilev K. Surface chemical functionalities affect the
42
43
44 behavior of human adipose-derived stem cells in vitro. *Appl. Surf. Sci.* 2013; 270: 473-
45
46
47
48 479.

49
50
51 66. Hulander M, Lundgren A, Berglin M, Ohrlander M, Lausmaa J, Elwing H. Immune
52
53
54 complement activation is attenuated by surface nanotopography. *International Journal*
55
56
57
58
59 of *Nanomedicine.* 2011; 6: 2653-2666.
60

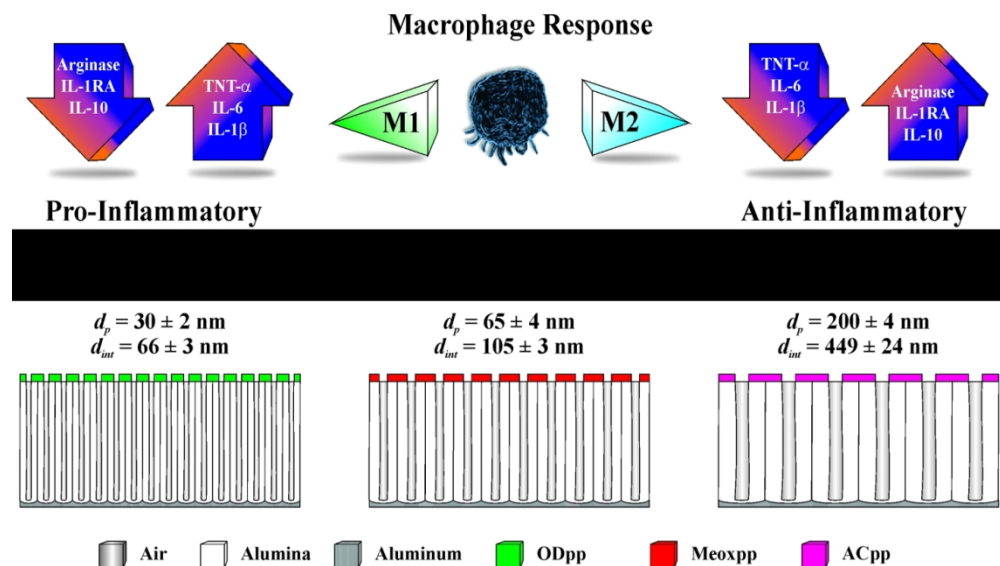
1
2
3 67. Bachhuka A, Delalat B, Ghaemi SR, Gronthos S, Voelcker NH, Vasilev K.
4
5

6
7 Nanotopography mediated osteogenic differentiation of human dental pulp derived
8
9
10 stem cells. *Nanoscale*. 2017; 9 (37): 14248-14258.
11
12

13
14 68. Liu X, Xie Y, Shi S, Feng Q, Bachhuka A, Guo X, She Z, Tan R, Cai Q, Vasilev K.
15
16

17 The co-effect of surface topography gradient fabricated via immobilization of gold
18
19
20 nanoparticles and surface chemistry via deposition of plasma polymerized film of
21
22
23
24 allylamine/acrylic acid on osteoblast-like cell behavior. *Appl. Surf. Sci.* 2019; 473: 838-
25
26

27
28 847.
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



Schematic representation of macrophage polarization (M1 and M2 phenotypes) on chemically and nanotopographically modified surfaces.

247x145mm (150 x 150 DPI)