Electronic Supplementary Material (ESI) for Environmental Science: Nano.

Supporting Information

Mechanistic insights into toxicity pathways induced by nanomaterials in Daphnia magna from analysis of the composition of the acquired protein corona

Laura-Jayne. A. Ellis and Iseult Lyncha

University of Birmingham, College of life and Environmental Sciences, Birmingham B15 2TT, United Kingdom

1. Methodology

1.1 Media and representative waters

Tables SI.1 and SI.1A provide a complete description of the chemical components used to make the three synthetic water compositions used in the present study.

Table SI.1 Media compositions					
Class I river water		**Class V river water**		**HH combo media**	
Compound	**Stock mg L^{-1}**	**Compound**	**Stock mg L^{-1}**	**Compound**	**Stock (g L^{-1})**
Calcium Sulfate Dihydrate	1.722	Calcium Sulfate Dihydrate	155.02	Calcium Chloride, Dihydrate	110.28
Calcium carbonate	2.002	Calcium carbonate	8.007	Magnesium sulphate heptahydrate	113.5
		Magnesium carbonate	45.517	Potassium phosphate dibasic	1.742
	µL L^{-1}		**µL L^{-1}**	Sodium nitrate	17
Calcium Nitrate Tetrahydrate*	5	Calcium Nitrate Tetrahydrate*	165	Sodium metasilicate nonahydrate	28.42
Sodium bicarbonate*	13.5	Sodium bicarbonate*	696.6	Boric acid	24
Calcium chloride*	7.5	Calcium chloride*	363.5	Potassium chloride	5.96
Potassium bicarbonate *	11.6	Potassium bicarbonate *	85.9	Sodium Bicarbonate	63
Magnesium nitrate Hexahydrate	48.9				
	mL L^{-1}		**mL L^{-1}**		
Natural organic matter (NOM)	1.84	Natural organic matter (NOM)	4.6		
pH	7.3-7.8	**pH**	7.6-7.8	**pH**	7.6-7.8
Ionic strength	10.12	**Ionic strength**	6	**Ionic strength**	11.07

*Stock solutions of 1 mol L^{-1}

Table SI.1A: Animate	
Compound	**Stock (g/100mL)**
lithium chloride	31
rubidium chloride	7
strontium chloride hexahydrate	15
sodium bromide	1.6
potassium iodide	0.33

*add 1mL of each to 1L

Add of each compound reported in Table SI.5A to prepare 1L stock solution of animate. From the 1L stock solution of animate, 1 mL is added to every 1 L of either the HH combo medium or the Class V water. To make the vitamin stock, aliquot 1.5 mL of the biotin and the B12 vitamins into 50 mL with 10 mg of Thiamine HCl (Table SI.5B). A total of 0.5mL from the vitamin stock is added to every 1 L of either the HH combo medium or the Class V water.

1.2 Nanomaterials and Characterization

All TiO_2 NMs were manufactured by Promethean Particles Ltd using flow reactor methods. Using this method, TiO_2 (anatase) NMs with a primary particle size of approximately 12 nm were produced. Functionalisation was carried out *in situ*, whereby capping agents (polymers) were pumped in downstream of the reactor. The capping agents were added at a concentration of 50 wt% relative to the mass of the TiO_2 NM product. The NMs were collected as aqueous suspensions and left to sediment over time, leaving a clear supernatant which was removed. The NMs were then washed by centrifugation and re-suspended in fresh deionized water, resulting in aqueous stock solutions at concentrations of ~2wt% TiO_2. These were shaken gently to reverse any settling prior to dispersion (Khan, 2020). Working stock solutions of 1000 mg L^{-1} were made by diluting the original suspensions into the required medium.

1.3 Experimental conditions

An EC_{30} was used as we wanted to show some toxicity effect in the pristine Ag NM exposures, which could be compared to the aged Ag NM studies which are hypothesized to show considerably less/no toxicity in comparison to the pristine counterparts (especially in the Class V water). Had we used an EC_{20} for the pristine NMs there would have been little to no effects on the daphnids to compare with the aged NMs, whereas the purpose of the study is to highlight that aging reduces toxicity, thus using a median range of EC_{30} allowed demonstration of modulated toxicity in the aged NMs. By contrast, we used a low EC concentration of the TiO_2, as previous studies identified that these NMs are much more toxic in chronic studies than in acute studies, such that initial reproductive experiments using the acute EC30 resulted in all organisms dying, (Ellis et al., 2020) and thus we chose a lower concentration for the study reported here or EC5threshold was over estimated.

1.4 Hard-corona isolation and identification of proteins

For mass spectrometry, samples were desalted using Millipore C18 ZipTips. Tips were prepared by pre-wetting in 100% acetonitrile and rinsing in 2x10 µL 0.1% trifluoroacetic acid, five times. The tip was then washed with 3x10 µL 0.1% trifluoroacetic acid to remove excess salts before elution of peptides with 10 µL of 50% acetonitrile/water/0.1% trifluoroacetic acid. Samples were dried down to remove the acetonitrile, and then re-suspended in 0.1% formic acid solution in water.

Samples were trapped on a µPrecolumn Cartridge, Acclaim PepMap 100 C18, 5 µm, 100A 300µm i.d. x 5mm (Dionex, Sunnyvale, CA USA) and separated in Nano Series™ Standard Columns 75 µm i.d. x 15 cm, packed with C18 PepMap100, 3 µm, 100Å (Dionex, Sunnyvale, CA USA). The gradient used was from 3.2% to 44% of 0.1% formic acid in acetonitrile over 30 minutes. Peptides were eluted directly (~ 350 nL min^{-1}) via a Triversa Nanomate nanospray source (Advion Biosciences, NY) into a QExactive HF (QEHF) mass spectrometer (Thermo Fisher Scientific). The data-dependent scanning acquisition is controlled by Xcalibur 4.0 software. The mass spectrometer alternated between a full FT-MS scan (m/z 360 – 1600) and subsequent high energy collision dissociation (HCD) MS/MS scans of the 20 most abundant ions. Survey scans were acquired in the QEHF with a resolution of 120,000 at m/z 200 and automatic gain control (AGC) $3x10^6$. Precursor ions were fragmented in HCD MS/MS with resolution set at 15,000 and a normalized collision energy of 28 keV. The ACG target for HCD MS/MS was $1x10^5$. The width of the precursor isolation window was 1.2 m/z and only multiply-charged precursor ions were selected for MS/MS. Spectra were acquired for 56 minutes with dynamic exclusion time of 20s. The MS and MS/MS scans were searched against the Uniprot database using Proteome Discoverer 2.1 (ThermoFisher Scientific).

Variable modifications are deamidation (N and Q), oxidation (M) and phosphorylation (S, T and Y). The precursor mass tolerance was 10 ppm and the MS/MS mass tolerance was 0.02Da. Two missed cleavages were allowed and identified proteins are accepted as a real hit protein with at least two high confidence peptides.

Water samples from the NM *Daphnia* incubations and samples from the NM free with *Daphnia* only (controls) were measured for total protein quantification using a Pierce BCA assay protein kit protein. A standard curve (Figure SI. 3) was created using the known standards of 0, 1, 2 and 3 mg/mL of bovine serum albumin concentrations against absorbance (set to 560 nm). Samples of unknown concentration could then be found by inserting the absorbance provided by the plate reader for each of the samples to calculate concentration.

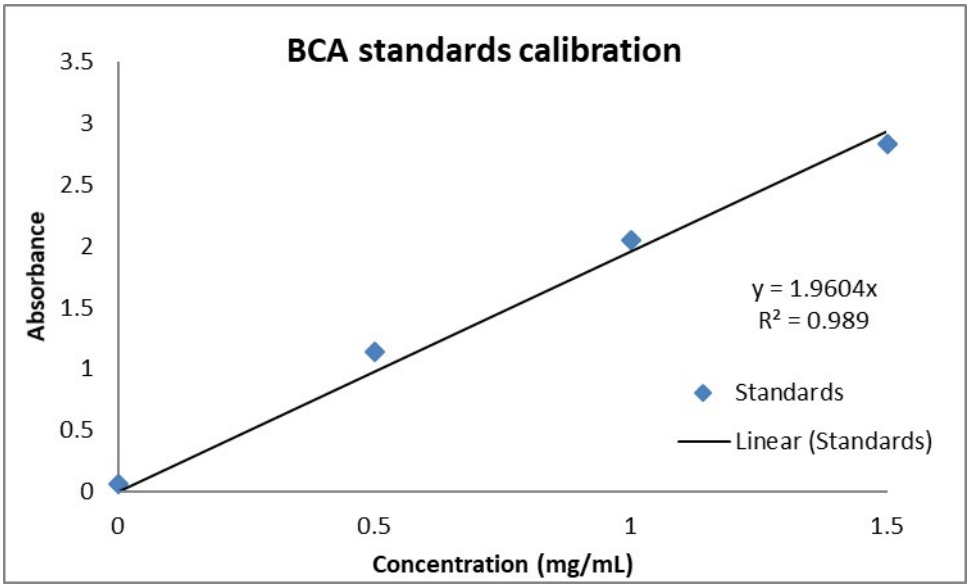

Figure SI.1: Absorbance calibration curve for the Pierce BCA assay kit using standards of 0, 1, 2 and 3 mg/mL of bovine serum albumin and their measured absorbance's to compare against the medium samples.

2 Results

2.1 Nanomaterials and Characterization

The pristine and aged TiO_2 dispersions in each of the media were visibly aggregated when imaged with TEM, although little difference was observed between the pristine and aged TiO_2 NM primary particle sizes. Ageing the Ag NMs in the various media had the least effect on the Ag_2S NMs, whereas the uncoated Ag was the most unstable leading to NM-NM hetero-agglomeration (Table SI.2).

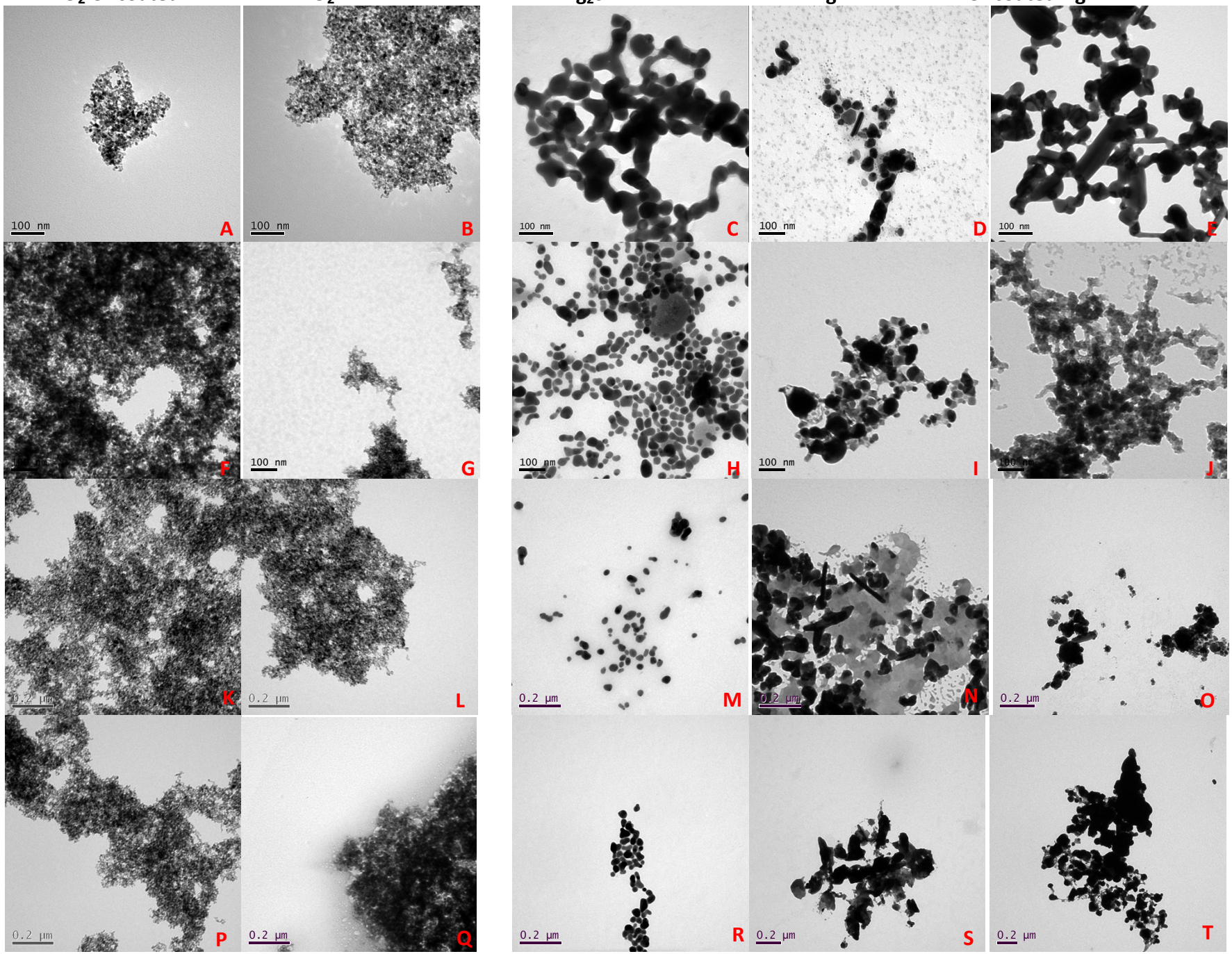
TiO2 Uncoated
TiO2 PVP
Ag2S
PVP Ag
Uncoated Ag
Pristine
6 months aged in HH combo
6 months aged in Class I water
6 months aged in Class V water
100 nm
0.2 µm
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T

Figure SI.2: Characterization of the NMs in the three media before and after 6 months ageing. A: Pristine uncoated TiO_2 in ultrapure water (UPW), **B:** Pristine PVP TiO_2 in UPW, **C:** Pristine Ag_2S in UPW, **D:** Pristine PVP Ag in UPW, **E:** Pristine uncoated Ag in UPW, **F:** Aged uncoated TiO_2 in HH combo media, **G:** Aged PVP TiO_2 in HH combo media, **H:** Aged Ag_2S in HH combo media, **I:** Aged PVP Ag in HH combo media, **J:** Aged uncoated Ag in HH combo media, **K:** Aged uncoated TiO_2 in Class I water, **L:** Aged PVP TiO_2 in Class I water, **M:** Aged Ag_2S in Class I water, **N:** Aged PVP Ag in Class I water and **O:** Aged uncoated Ag in Class I water, **P:** Aged uncoated TiO_2 in Class V water, **Q:** Aged PVP TiO_2 in Class V water, **R:** Aged Ag_2S in Class V river water, **S:** Aged PVP Ag in Class V water and **T:** Aged uncoated Ag in Class V water. The pristine and aged TiO_2 dispersions in each of the media were visibly aggregated when imaged with TEM, although little difference was observed between the pristine and aged TiO_2 NM primary particle sizes.

Table SI.2: Characterization of Ag and TiO_2 NMs using TEM and DLS

Identifier	Freshly dispersed NMs TEM individual particle size (nm)	Aged NMs in HH combo TEM individual size (nm)	Aged NMs in Class I TEM individual size (nm)	Aged NMs in Class V TEM individual size (nm)	Freshly dispersed NMs DLS particle/aggregate size (nm)	Aged NMs in HH combo DLS particle/aggregate size (nm)	Aged NMs in Class I river water DLS particle/aggregate size (nm)	Aged NMs in Class V river water DLS particle/aggregate size (nm)
Ag Uncoated	61 ± 36	105 ± 228	51 ± 35	95 ± 111	323 ± 73	7363 ± 1054	867 ± 642	171 ± 22
Ag PVP	18 ± 11	37± 20	74 ± 52	105 ± 102	44 ± 2	129 ± 22	160 ± 51	291 ± 141
Ag_2S	44 ± 14	38 ± 15	40 ± 16	49 ± 15	219 ± 17	145 ± 2	173 ± 6	309 ± 360
TiO_2 Uncoated	9 ± 2	10 ± 3	10 ± 3	18 ± 77	207 ± 11	305 ± 139	508 ± 42	8001 ± 1453
TiO_2 PVP	9 ± 2	10 ± 3	11 ± 3	15 ± 4	311 ± 43	305 ± 176	202 ± 74	225 ± 79

Results

2.3 Characterisation of protein corona compositions

Table SI. 3 shows the total protein concentrations from the water samples from the NM + *Daphnia* incubations and samples from the NM free + *Daphnia* (controls) determined by BCA assay after 7 days of incubation. The results show that the untreated controls have a baseline concentration of secreted proteins in the water as a result of the filter feeding mechanisms. In addition, the NM treatments lead to increased daphnid secretions, with the highest secretions corresponding to those NMs found to be most toxic in previous studies.

Note samples were no samples for the control and pristine NM in the Class I water.

Table SI.3: BCA assay quantification of total protein associated with the NMs

Samples in HH combo media	Total concentration of protein µg / mL	Samples in Class V water	Total concentration of protein µg / mL	Samples in Class I water	Total concentration of protein µg / mL
Control HH combo	40	Control Class V	45	Control Class I	43
Bulk Ag HH combo	53	Bulk Ag Class V	48	Bulk Ag Class I	48
PVP only HH combo	57	PVP only Class V	66		
TiO_2 Uncoated freshly dispersed in HH combo	51	TiO_2 Uncoated pristine Class V	44	TiO_2 uncoated in aged Class I	39
TiO_2 uncoated aged HH combo	43	TiO_2 uncoated aged in Class V	71		
TiO_2 PVP freshly dispersed in HH combo	47	TiO_2 PVP freshly dispersed in Class V	46	TiO_2 PVP aged in Class I	50
TiO_2 PVP aged in HH combo	75	TiO_2 PVP aged in Class V	50		
Uncoated Ag freshly in HH combo	59	Uncoated Ag freshly dispersed in in Class V	47	Uncoated Ag aged in Class I	44
Uncoated Ag Aged in HH combo	43	Uncoated Ag aged in Class V	57		
PVP Ag freshly dispersed in HH combo	43	PVP Ag freshly dispersed in in Class V	50	PVP Ag aged in Class I	51
PVP Ag aged in HH combo	61	PVP Ag aged Class V	57		
Ag_2S freshly dispersed in HH combo	43	Ag_2S freshly dispersed in Class V	59	Ag_2S aged in Class I	37
Ag_2S aged in HH combo	60	Ag_2S aged in Class V	46		

LC-MS analysis identified the total number of proteins present in the NM coronas for each condition and the secreted proteins by the daphnids in the absence of NMs in the media. Proteins can be qualitatively identified by their molecular weight (MW). The MW of the proteins were classified into groups for each NM condition (and control) as shown in Figure SI.5.

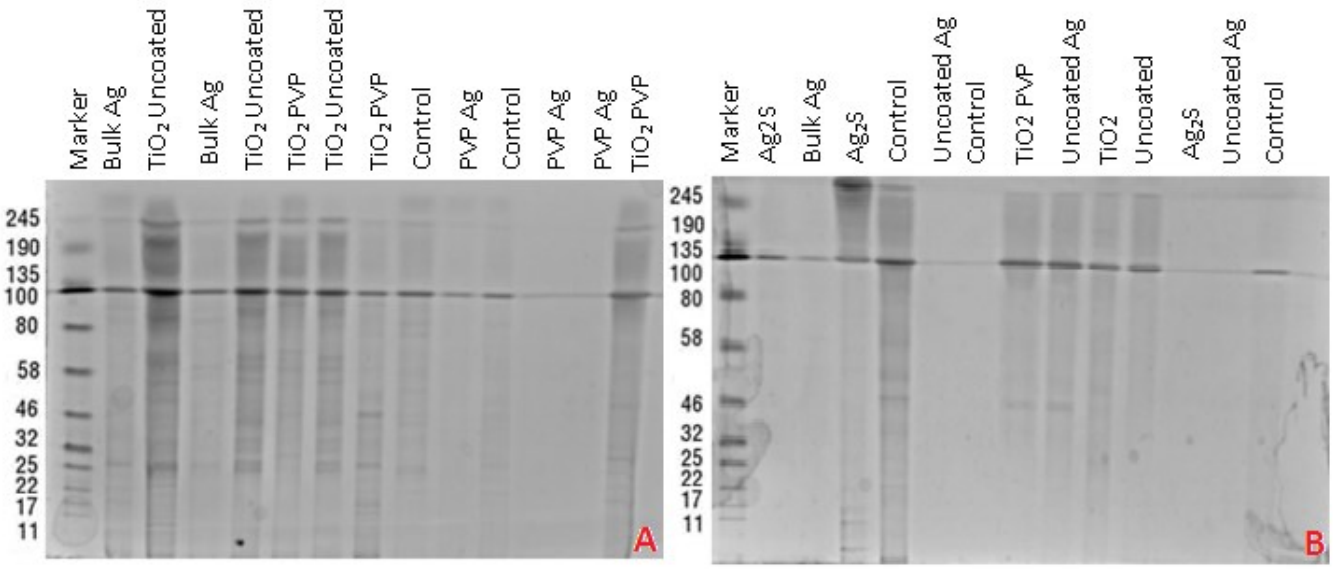

Figure SI.3: Examples of 12.5% polyacrylamide gel electrophoresis (PAGE) using Coomassie blue (0.25%) and silver stain using 20 µL of eluted protein per well. To the left of each gel is a protein band standard ladder (p77125 bioLabs), with samples loaded to the right of the ladder. Samples are from HH combo exposed to the pristine NMs, which each NM added in triplicate randomly.

Characterisation of protein corona associated with the GO molecular and biological pathways

Using the Uniprot database (https://www.uniprot.org/) and the quick GO gene ontology and gene annotations via The European Bioinformatics Institute: proteins that were uniquely expressed by Daphnids in response to the pristine and/or aged NMs in the various water conditions (identified by LC-MS), were searched to identify their gene ontology (GO) terms which displays the biological processes of the ancestor chart. Below are some examples of these ancestor charts for particular proteins of interest.

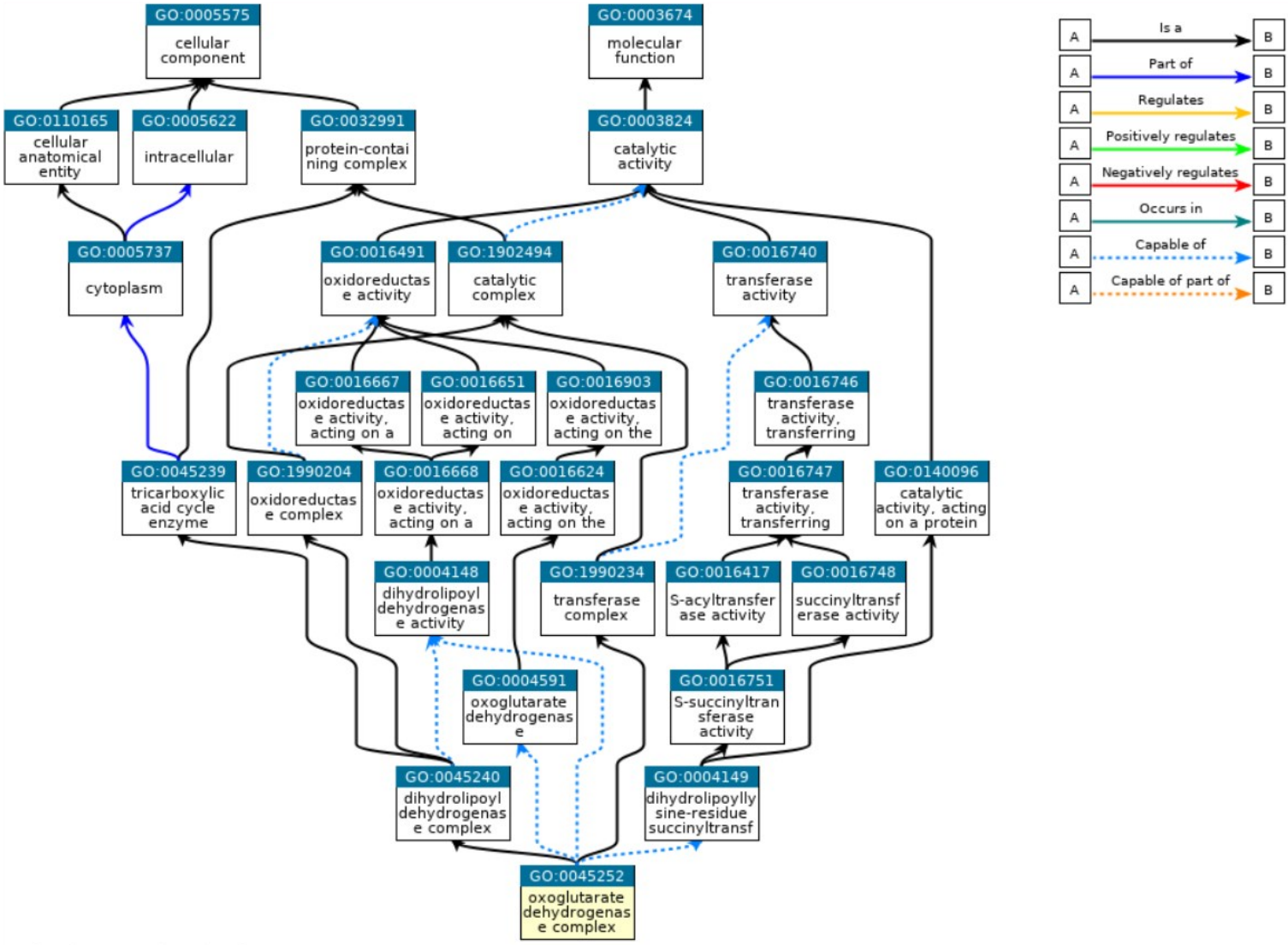

Figure SI.4: The 2-oxoglutarate dehydrogenase E1 protein was unique to the corona of aged PVP Ag NMs in the Class V water. Image source via The European Bioinformatics Institute: https://www.ebi.ac.uk/QuickGO/term/GO:0045252

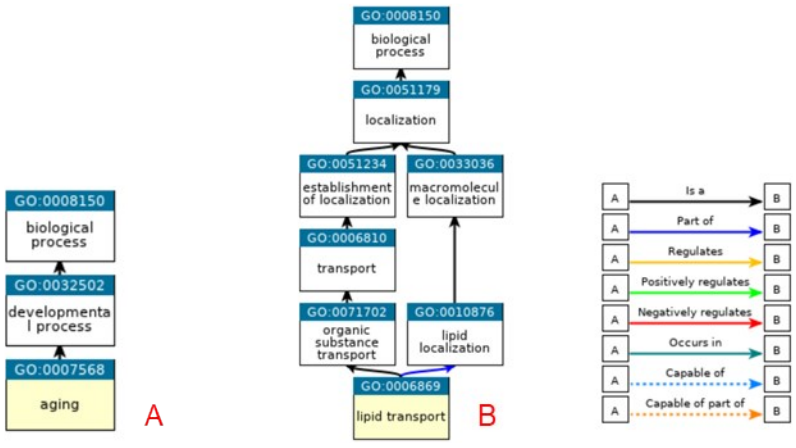

Figure SI.5: Ancestor charts for the GO pathways that were associated with the apolipoprotein D were ageing **(A)** and lipid transport **(B).** Apolipoprotein D was unique to the corona of the aged PVP TiO_2 NMs in the Class V water. Image source European Bioinformatics Institute: **(A)** https://www.ebi.ac.uk/QuickGO/term/GO:0007568 and **(B)** https://www.ebi.ac.uk/QuickGO/term/GO:0006869

The following Figures (SI.8-10) show the GO ancestor charts for pathways which have a high degree of species conservation that were linked to lipid transport, antioxidant processes and developmental processes.

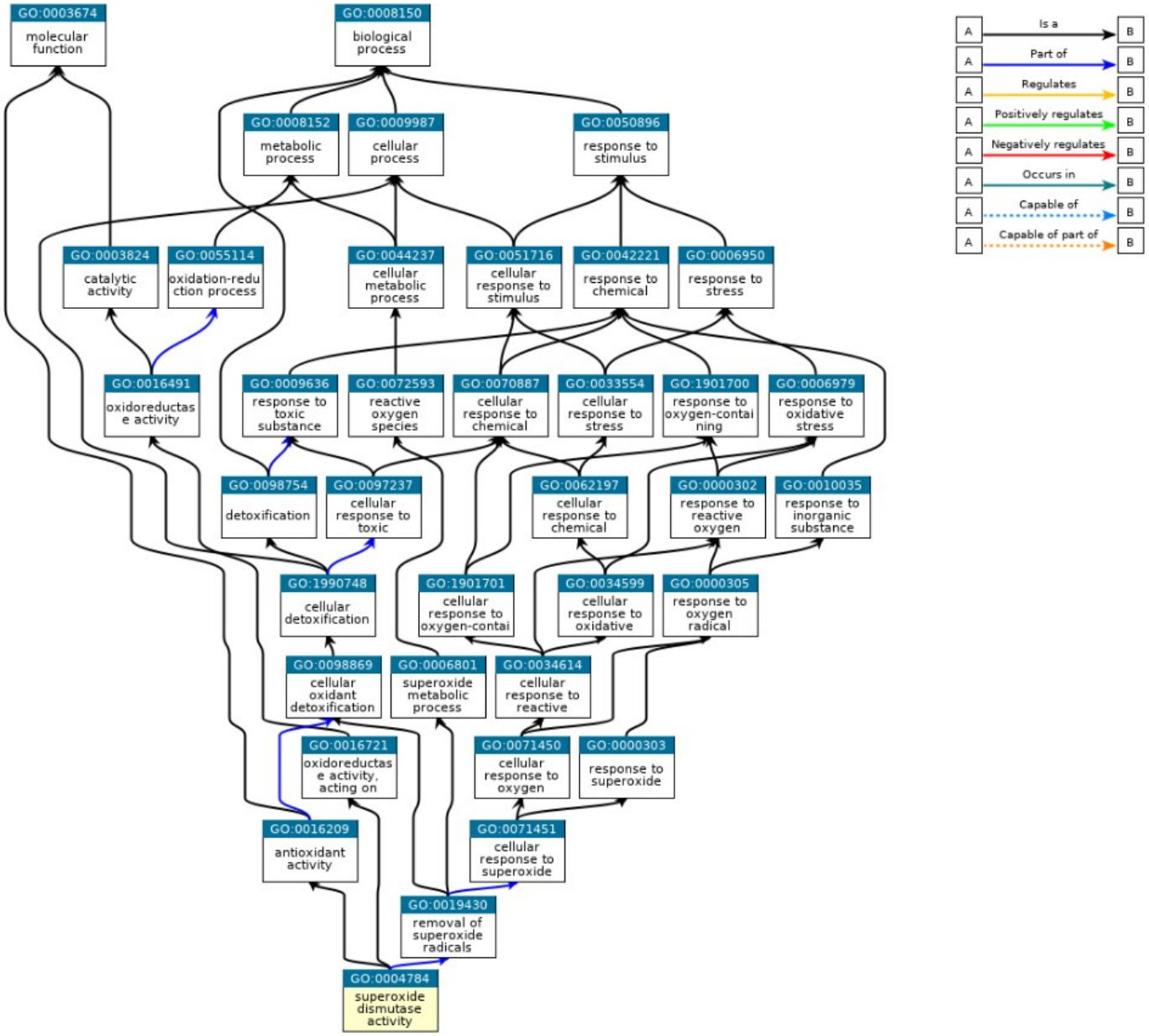

Figure SI.6: Ancestor chart for the GO pathways that were associated with copper-zinc superoxide dismutase activity. Identified in the coronas of the pristine PVP Ag NMs in HH combo media; pristine bulk Ag and pristine PVP Ag NMs in Class V water. Image source via: The European Bioinformatics Institute: https://www.ebi.ac.uk/QuickGO/term/GO:0004784

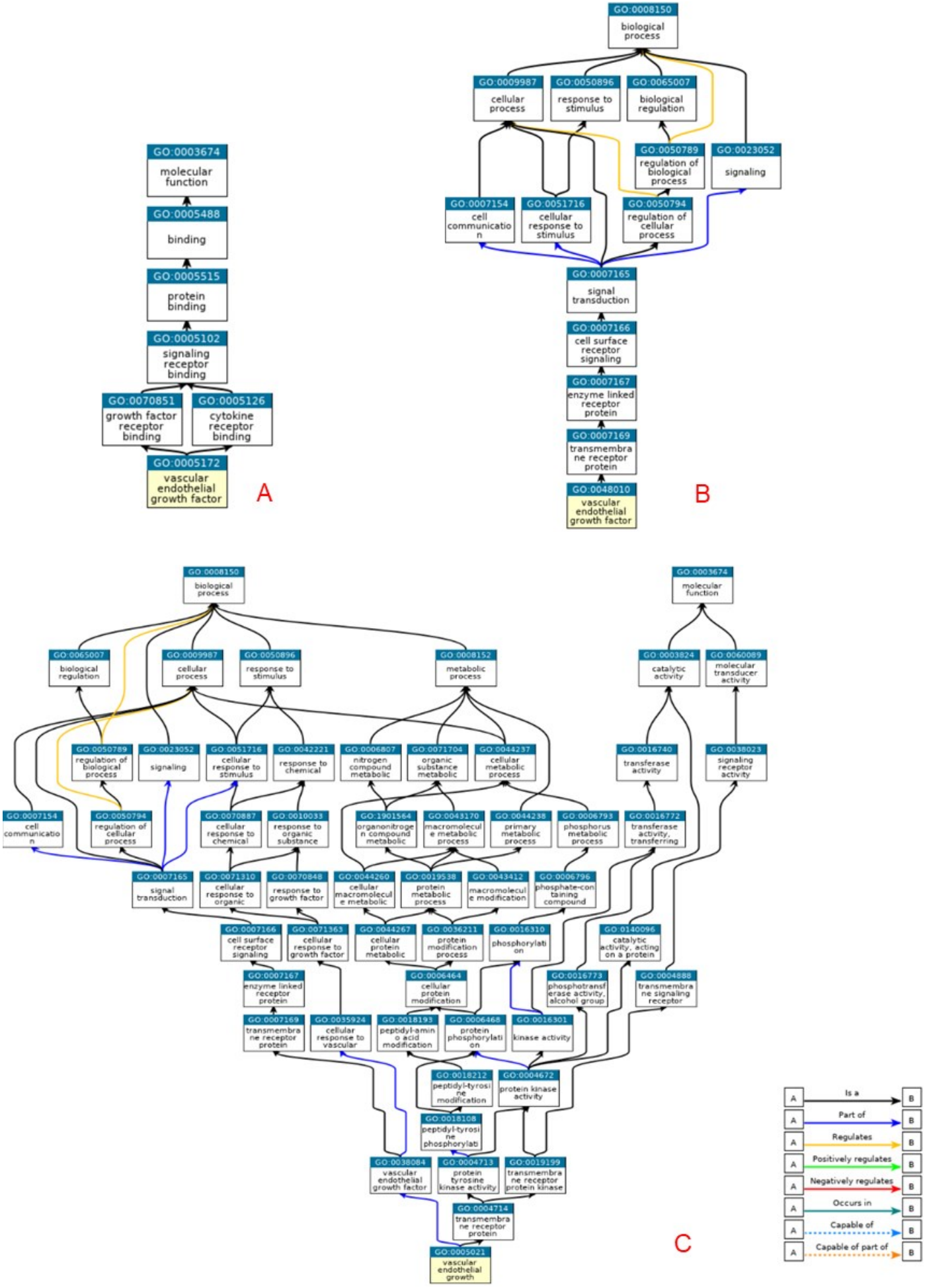

Figure SI.7: Ancestor chart for the GO pathways that were associated with the vascular endothelial growth factor receptor proteins **(A)** describes the binding processes **(B)** signalling pathways and **(C)** the receptor. Vascular endothelial growth factor receptor proteins were found in the coronas of aged PVP Ag and aged TiO_2 PVP NMs both in HH combo media.

Image source via: The European Bioinformatics Institute: (A) https://www.ebi.ac.uk/QuickGO/term/GO:0005172 (B) https://www.ebi.ac.uk/QuickGO/term/GO:0048010 (C) https://www.ebi.ac.uk/QuickGO/term/GO:0005021

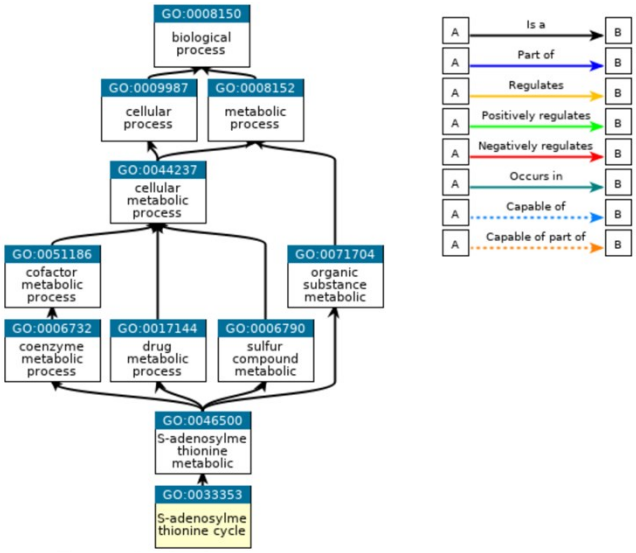

Figure SI.8: Ancestor chart for the GO interacting cycle of the biological processes that were associated with S-adenosylmethionine. These proteins were found in the coronas of the following NMs: Aged TiO_2 PVP and aged uncoated Ag in both the HH combo and Class V water, aged Ag_2S, aged PVP Ag, Aged TiO_2 uncoated and aged uncoated Ag all in Class I water. Image source via: The European Bioinformatics Institute: https://www.ebi.ac.uk/QuickGO/term/GO:0033353

Figures SI.9-11 below describe the associated GO terms relating to the biological and molecular pathways for each of the identified proteins in the NM coronas and the NM-free media controls. Note, not all the protein IDs are shown on the Y axis due to character spacing. Some proteins were also involved in multiple pathways, and therefore are only listed as their primary GO terms for comparison as described at www.uniprot.org. A full list of the protein IDs are located in Table SI.4.

Figure SI.9: GO terms for protein identified in the surface coronas of the uncoated Ag NMs for each NM condition (pristine Vs ages) and each medium (HH combo, Class I and Class V).

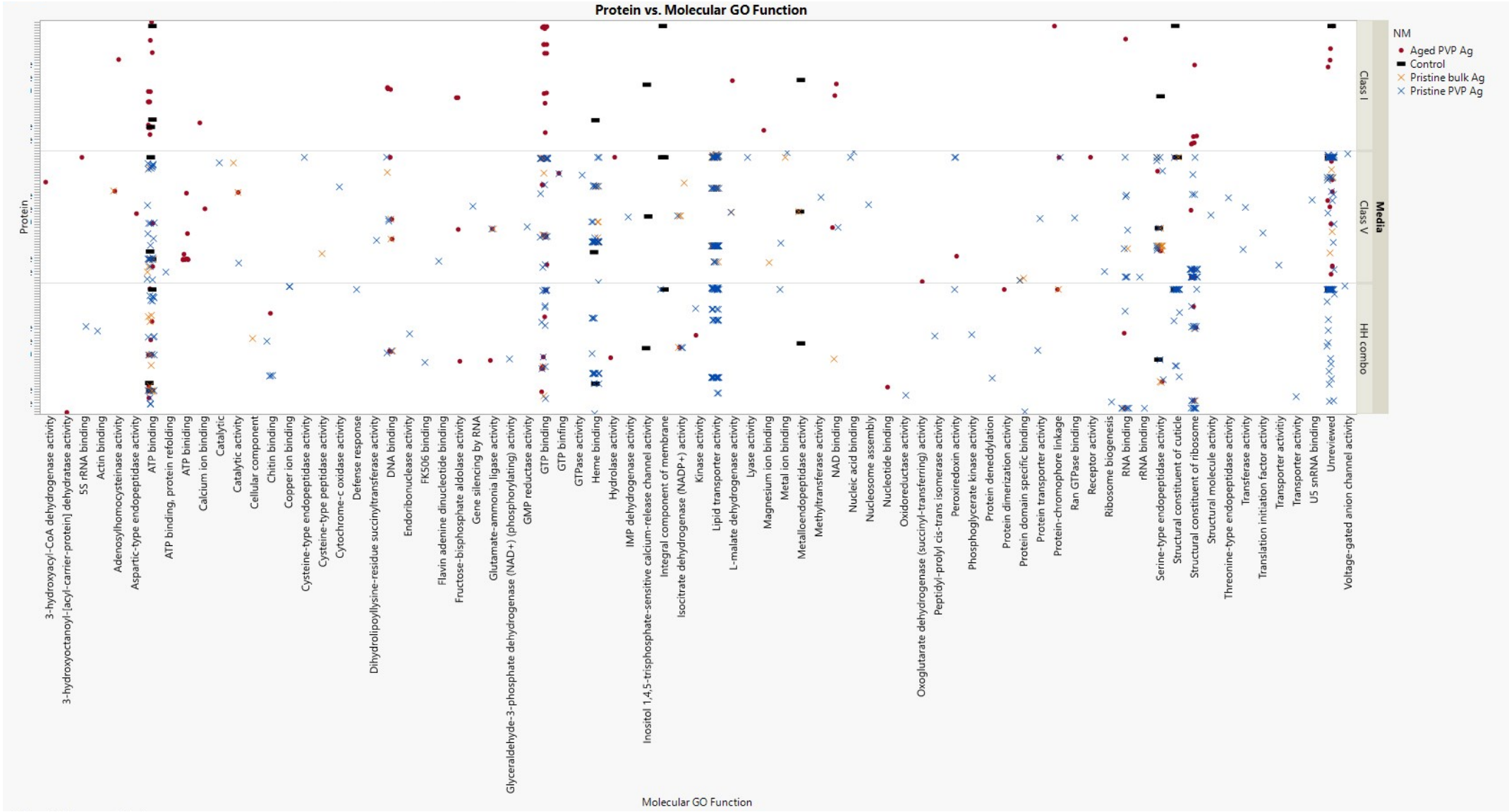

Figure SI.10: GO terms for protein identified in the surface coronas of the PVP Ag NMs for each NM condition (pristine Vs ages) and each medium (HH combo, Class I and Class V).

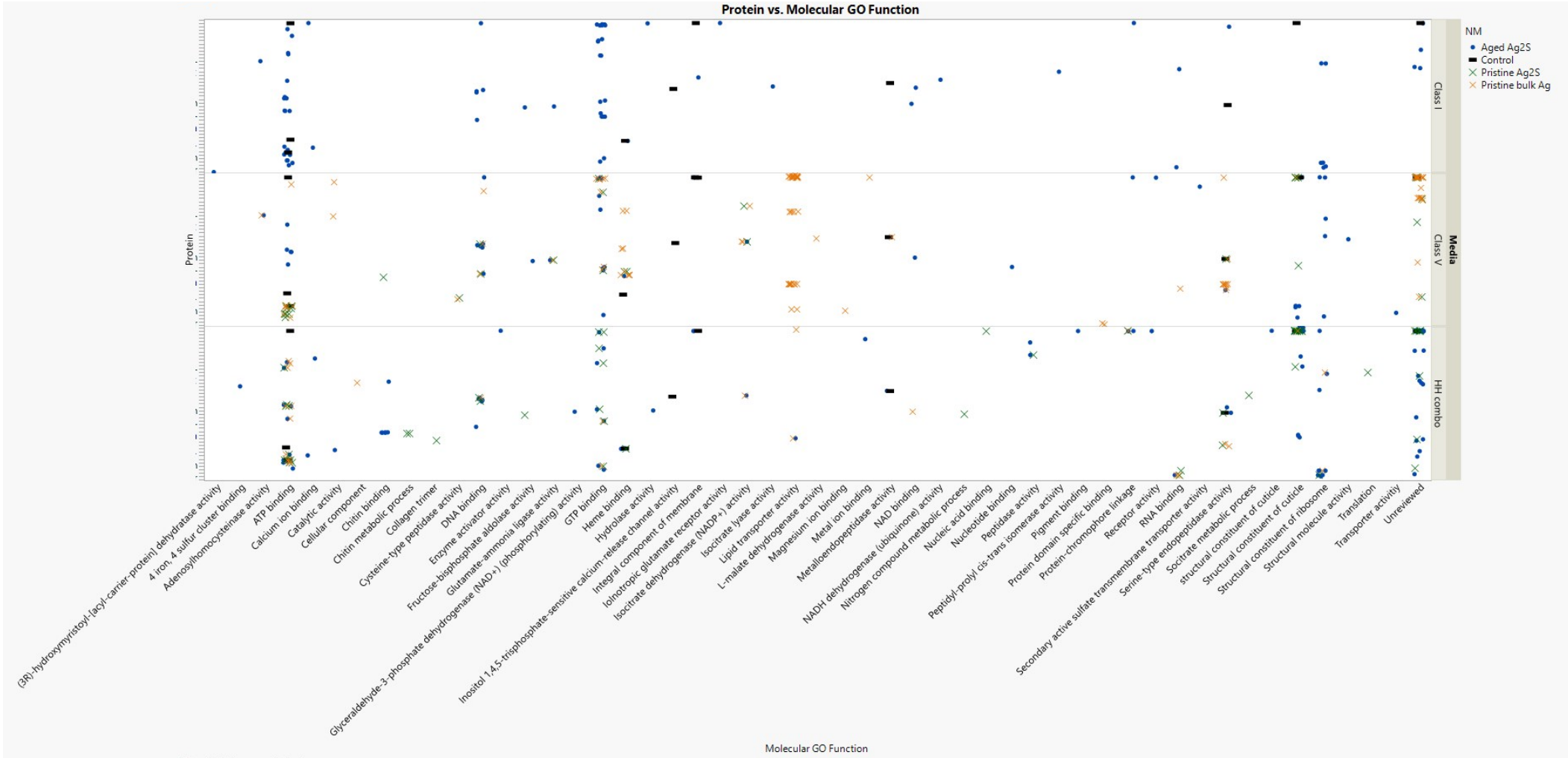

Figure SI.11: GO terms for protein identified in the surface coronas of the Ag_2S NMs for each NM condition (pristine Vs ages) and each medium (HH combo, Class I and Class V).

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