Supplementary Information

Identification of peptide coatings that enhance diffusive transport of nanoparticles through the tumor microenvironment

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Table S1. Oligonucleotides designed for cloning of the selected peptide sequences from phage display and the control

Clones	**Reverse complement oligonucleotide sequences**
C1 (CHPRSTPNC)	5'- AAC TGC AAG CTT TTA GCA ATT CGG AGT AGA CCT AGG ATG GCA AGA ATT CGG ATC CCC GAG CAT- 3'
C2 (CVLADGNAC)	5'- AAC TGC AAG CTT TTA GCA CGC ATT CCC ATC AGC CAA CAC GCA AGA ATT CGG ATC CCC GAG CAT-3'
C3 (CRLEDGTLC)	5'- AAC TGC AAG CTT TTA GCA AAG AGT ACC ATC CTC CAG ACG GCA AGA ATT CGG ATC CCC GAG CAT- 3'
C4 (CKPGDGGPC)	5'- AAC TGC AAG CTT TTA GCA AGG ACC TCC ATC TCC AGG TTT GCA AGA ATT CGG ATC CCC GAG CAT- 3'
C5 (CRPGSGGAC)	5' - AAC TGC AAG CTT TTA GCA AGC ACC TCC AGA TCC AGG TCT GCA AGA ATT CGG ATC CCC GAG CAT- 3'
Control (CDIEAEEEC)	5'- AAC TGC AAG CTT TTA GCA TTC CTC TTC AGC TTC AAT ATC GCA AGA ATT CGG ATC CCC GAG CAT- 3'

Table S2. Oligonucleotides designed for alanine mutagenesis of the selected clone, P4-phage

Negative (CAPGDGGPC)	Sense: 5'-----AAT TCT TGC GCT CCT GGA GAT GGA GGT CCT TGC TAA A---3'
	Asn: 5'-----AG CTT TTA GCA AGG ACC TCC ATC TCC AGG AGC GCA AG---3'

Positive (CKPGAGGPC)	Sense: 5'----AAT TCT TGC AAA CCT GGA GCT GGA GGT CCT TGC TAA A----3'
	Asn: 5'---- AG CTT TTA GCA AGG ACC TCC AGC TCC AGG TTT GCA AG----3'
Hydro-Neutral (CKAGDGGAC)	Sense: 5'--- AAT TCT TGC AAA GCT GGA GAT GGA GGT GCA TGC TAA A---3'
	Asn: 5'---AG CTT TTA GCA TGC ACC TCC ATC TCC AGC TTT GCA AG ----3'
Hydrophobic (CKAADAAAC)	Sense: 5'---- AAT TCT TGC AAA GCT GCA GAT GCT GCA GCC TGC TAA A---3'
	Asn: 5'-----AG CTT TTA GCA GGC TGC AGC ATC TGC AGC TTT GCA AG ---3'
Interchange (CDPGKGGPC)	Sense: 5'---AAT TCT TGC GAT CCT GGA AAA GGA GGT CCT TGC TAA A ----3'
	Asn: 5'----AG CTT TTA GCA AGG ACC TCC TTT TCC AGG ATC GCA AG ----3'
Adjacent (CGKDPGPGC)	Sense: 5'---AAT TCT TGC GGA AAA GAT CCT GGA CCT GGT TGC TAA A----3'
	Asn: 5'-----AG CTT TTA GCA ACC AGG TCC AGG ATC TTT TCC GCA AG ----3'

Script S1. MATLAB script for Particle tracking

```
clear all
%create cells to store data for all the images in the file
I=cell(1,300);
J=cell(1,300);
b=cell(1,300);
pk=cell(1,300);

%Read images
for i=1:300
    if i>=1 && i<=9
    I{i}=double(imread(sprintf('Image_T000%d.tif',i)));
    elseif i>=10 && i<=99
            I{i}=double(imread(sprintf('Image_T00%d.tif',i)));
    elseif  i>=100 && i<=300
            I{i}=double(imread(sprintf('Image_T0%d.tif',i)));
    end
    %doing this because RGB image, last column access different colors
    J{i}(:,:)=I{i}(:,:,1);
    whos J{i};
    %colormap('gray'), imagesc(J)
    b{i}=bpass(J{i},0,size of particle,cut-off intensity);
    pk{i}=pkfnd(b{i},minimum value of intensity of local maxima, size)
    max(max(b{i}));
    % N x 4 array containing, x, y and brightness for each feature
    % out(:,1) is the x-coordinates
    % out(:,2) is the y-coordinates
    % out(:,3) is the brightnesses
    % out(:,4) is the sqare of the radius of gyration
```

```
    cnt{i}=cntrd(b{i},pk{i},size+2);
    lencnt(1,i)=size(cnt{i},1);
end
lencnt2=lencnt';

%Extract only x- and y-coordinates of the particles
B= cell2mat(cnt')
sizB=size(B,2);
k=0;
for i=1:size(lencnt2,1)
    if i~=1
    k=k+lencnt2(i-1);
    end
    B(k+1:k+lencnt2(i),sizB+1)=i;
end
B

%Tracking of particles
tr = track(B, maximum displacement)
T= table(tr);
%Export tracking information to excel
filename='trackeddata.xlsx';
writetable(T,filename,'Sheet',1)
```

Supplementary Note 1. In silico analysis evaluates the overall physicochemical properties of the thirty most frequent peptide sequences

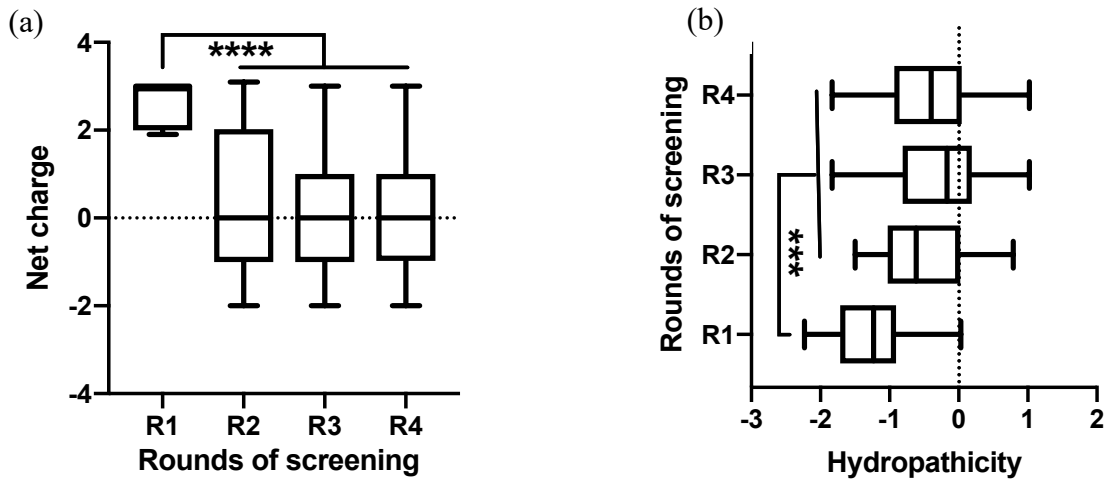

Figure S1. Physicochemical properties of phage after selection rounds. Average (a) net charge at pH 7 and (b) hydropathicity of the top thirty frequent sequences diffused over four rounds of screening (R1, R2, R3, and R4) analyzed from NGS data. Data represents median, IQR. One-way analysis of variance (ANOVA) with Dunnett's T3 multiple comparisons test ($p<0.0001$).

The net charge of the top thirty frequent peptides from each round of screening was calculated, and their overall net charge was plotted (**Figure S1a**). From the whisker plot, the sequences from the first round (denoted as R1) have a median charge of +3, and all analyzed sequences were basic. In subsequent rounds (denoted as R2 – R4), there is a decrease in the median net charge compared to R1 ($p < 0.0001$). The median net charge changes from +3 in R1 to 0 in R2 to R4, and this finding suggests that during screening, there is a selection for net neutral charge peptides that could permeate through the *in vitro* tumor ECM. While the tumor ECM has overall net negative charge, the collagen fibers has a slightly positive surface charge density of 0.002 C/m^2 and hyaluronic acid has a highly negative surface charge density of -0.10 C/m^2(1). Presence of both of these components may result in localized charge patches, which makes the ECM an effective and complex electrostatic filter that is able to restrict the diffusion of both positively and

negatively charged molecules(2-4). Due to the role of these electrostatic interactions, neutral molecules can penetrate more rapidly compared to their charged counterparts.

Next, we characterized the overall hydrophobicity of our most abundant sequences. The Grand Average of Hydropathy (GRAVY), which is a score of average hydrophobicity estimated from an amino acid sequence, was calculated for each peptide from the top thirty sequences in each round and replicate (**Figure S1b**)(5). A negative GRAVY score represents an amino acid sequence that is hydrophilic, whereas a positive score indicates the amino acid sequence is hydrophobic(6). Most of peptides from the R1 eluate are hydrophilic, ranging from a minimum hydropathicity score of -2.23 to a maximum of 0.03. With each successive round, there is an increase in the minimum and maximum GRAVY score; the minimum and maximum hydropathy scores range from -1.83 to 1.02 in the R3 and R4 eluates. The net median hydropathy increases significantly from -1.23 in R1 to -0.4 in R4 ($p = 0.0002$). This suggests there are less hydrophilic amino acids of selected peptides that permeate through the tumor-like ECM, but generally the sequences are net hydrophilic. This change could be attributed to the loss of basic amino acids during selection and the increased presence of glycines. Glycines have been used as flexible linkers to provide greater conformational stability; however they contribute to the hydrophobicity of a sequence(7). Selection of net neutral charged peptides over rounds of screening (SI Figure 1a) also indicates a reduction in the number of both acidic and basic amino acid residues. Reduction in these amino acids simultaneously resulted in the decrease of the net hydrophilicity of the peptides in higher rounds.

Table S3. Top-thirty frequent peptide sequences from fourth round of screening of both replicates with their consideration for sequence alignment

Number	**Replicate 1**	**Considered**	**Replicate 2**	**Considered**
1[#]	CTLRVWTTC	Yes	CTLRVWTTC	Yes
2	CKSPSYGAC*KLLATQ	No	**CHPRSTPNC**	Yes
3	CRYSLLHHC	Yes	CKLADGKPC	Yes
4	CPGPSEQPC	Yes	CSPSSDRDC	Yes
5	CVATPSLVC*KLLAPL	No	CTIDGKTPC*KLLALRP	No
6	**CHPRSTPNC**	Yes	CKGGRASKC	Yes
7	CGPNDTHPC	Yes	**CVLADGNAC**	Yes
8	CPVNGSAAC	Yes	CRDSNGNPC	Yes
9	CTIDGKTPC*KLLALR	No	CGKGSSQVC	Yes
10	CTKDCSA*	No	CNLASGSMC	Yes
11	CKTGTSADC	Yes	CKLDSGEAC	Yes
12	CHQSSRPLC*KLLALPP	No	CVPGLDNEC	Yes
13	CV*GPKTG	No	CSHTSDGSC	Yes
14	CEPFGGRDC	Yes	CPHDATNPC	Yes

15	CNPASLSEC*KLLAHIL	No	CTNC*VATC*KLLAPT	No
16	CKADGKARC	Yes	CTPSTGGSC	Yes
17	**CRLEDGTLC**	Yes	CTDGSTTPC*KLLANT	No
18	CFSD*TTI	No	CRTPDGRAC	Yes
19	**CVLADGNAC**	Yes	CGPNRNQRC	Yes
20	CHLANGEAC	Yes	CGTSLEGKC	Yes
21	CAPGTIC*KLLAT	No	CTSSSRQKC	Yes
22	CDPMNPGSC	Yes	CKAGSADIC	Yes
23	CYTSDGNPC	Yes	CGPMQGREC*KLLANK	No
24	CANC*ADAVLKA	No	CRLPDGGAC*KLLARP	No
25	CQDC*GVR	No	CRNGVGGAC	Yes
26	CGTDPTLPC*KLLAPRP	No	CQPGEC*EC	No
27	CGPMQGREC*KLLANKT	No	CMRNARTKC	Yes
28	CEWEVWYRC	Yes	**CRLEDGTLC**	Yes
29	CALTGEC	No	CRTIPGEDC	Yes
30	CSSGPDGVC	Yes	CQPGLGKEC	Yes

[#] Peptide not used due to the challenges in genetically engineering it on the surface of T7 bacteriophage.

Table S4. Diffusivity of different T7 phage in PBS measured by dynamic light scattering

Peptide Name	Diffusivity in PBS at 25°C (m^2/sec)
WT	$(5.30\pm0.09)\times10^{-12}$
P1-phage	$(4.82\pm0.02)\times10^{-12}$
P2-phage	$(6.27\pm0.12)\times10^{-12}$
P3-phage	$(6.07\pm0.01)\times10^{-12}$
P4-phage	$(6.06\pm0.02)\times10^{-12}$
P5-phage	$(5.74\pm0.01)\times10^{-12}$
Control-phage	$(5.75\pm0.08)\times10^{-12}$

Determination of diffusion coefficient of clones through tumor ECM using microchannel diffusion assay

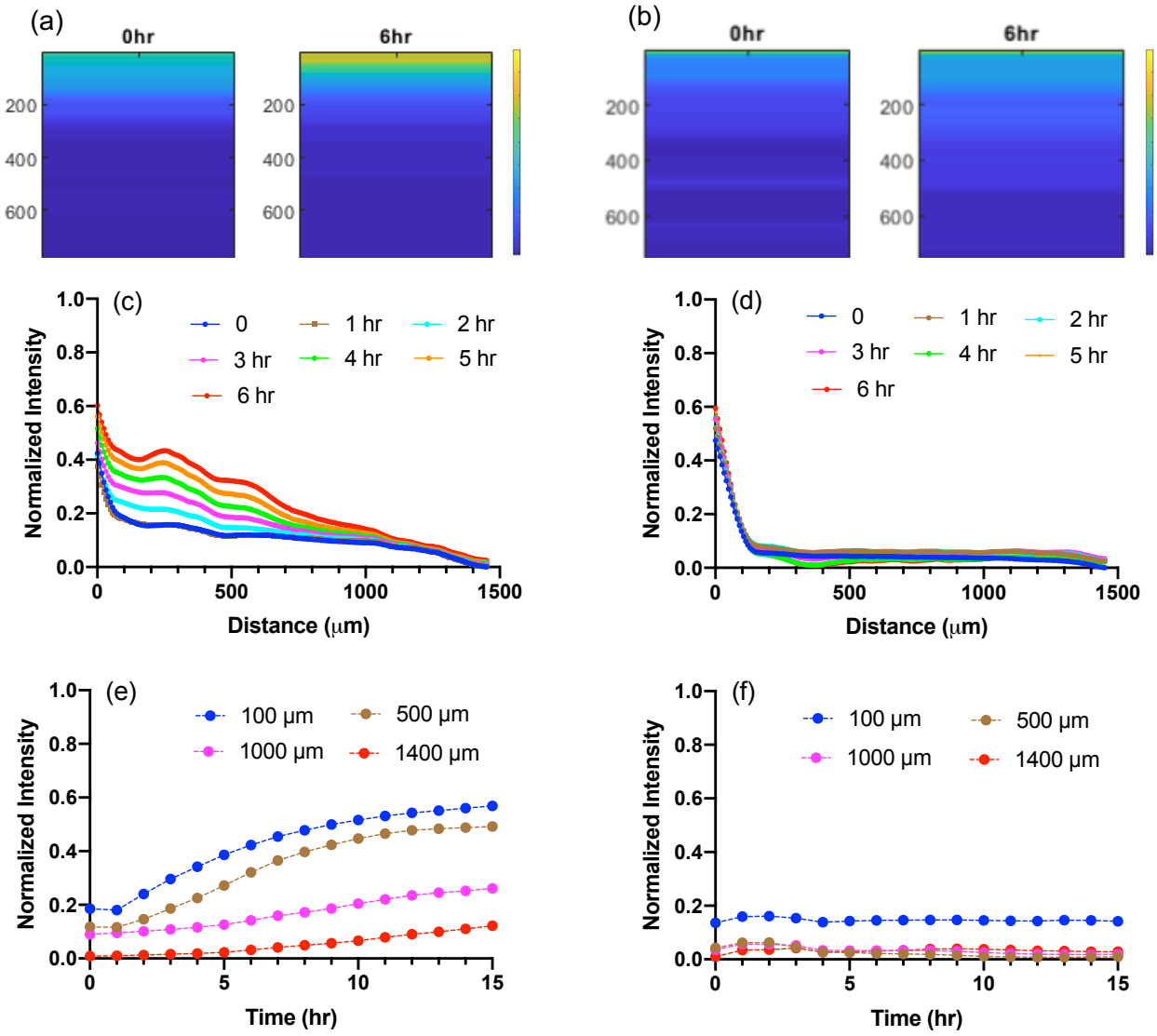

Figure S2. Representative images obtained from MATLAB analysis for μ-slide diffusion assay are compared for P4-phage and the negative control-phage. The colormaps generated in Matlab are showing the intensity profile of the fluorescently-labeled (a) P4-phage and (b) negative control-phage at initial time point (time 0) and after 6 hours of diffusion through the microchannel. P4-phage penetrates a longer distance in 6 hours compared to the control-phage. The normalized fluorescent intensity is plotted against the channel length from the input reservoir up to 6 hours with 1 hour time interval for (c) P4-phage and (d) control-phage. Although at the inlet reservoir the normalized intensity is ~0.6 for both of the clones initially, the normalized intensity dropped to less than 0.1 for the negative control-phage within 100 μm, and it remained the same even at higher time points. However, P4-phage penetrated up to ~1.45 mm of the channel length and the intensity increased with time for each penetration depth. Similarly, the evolution of normalized intensity with time is studied at fixed penetration distance up to initial 1.4 mm for (e) P4-phage

and (f) control-phage. The normalized intensity increases slightly in the first 1 hour, but remained constant throughout the higher time points up to 15 hours, and the value remained within 0.2 throughout for the control-phage. On the other hand, the normalized intensity increased steadily to 0.6 with time for all the channel lengths reported for P4-phage.

Supplementary Note 2. Hydrophilic and net neutral clone, C4 diffuses faster than other selected clones through tumor ECM using a transwell assay

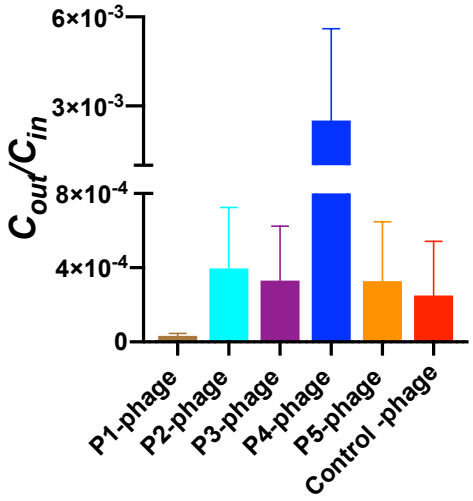

Figure S3. Higher output titer (C_{out}) of the clone, P4-phage is collected after 1 hr. of diffusion through the transwell compared to other selected clones and the negative control-phage while keeping the phage input (C_{in}) to be the same.

Diffusion of the selected clones is validated using bulk transwell diffusion assay in which the clones are made to permeate through the *in vitro* tumor ECM. A 1mm thick tumor ECM is prepared in the insert of a 3.0 µm pore size polyester membrane 24-well transwell (Corning) as described in the phage library screening section. 70 µL of 4.6×10^6 pfu/µL clone is added at the top of the ECM as input in the donor chamber of the transwell. The clones are rested on the ECM for 15 minutes before the diffusion assay to account for the unhindered diffusion of the clones because of the ECM leakiness. Then the insert is transferred to a new receiving chamber filled with 600 µL of PBS to equilibrate the hydrostatic pressure and allow the clone to diffuse through the ECM. 50 µL samples are collected at time intervals of 10, 20, 30, 40 and 50 minutes, and an equal volume of fresh PBS 1× is replenished. Temperature is maintained at 37 °C throughout the diffusion experiment. After 1 hour, all the eluates from the receiving chamber is collected. The output titer of the clones is quantified using standard double-layer plaque assay to compare the

output titer of the clones and the negative control-phage. Comparison of the output titer per the constant input titer (**Figure S3**) shows a hydrophilic and net neutral surface charge clone, P4-phage has higher penetration through the tumor ECM compared to all the other selected clones and the control-phage.

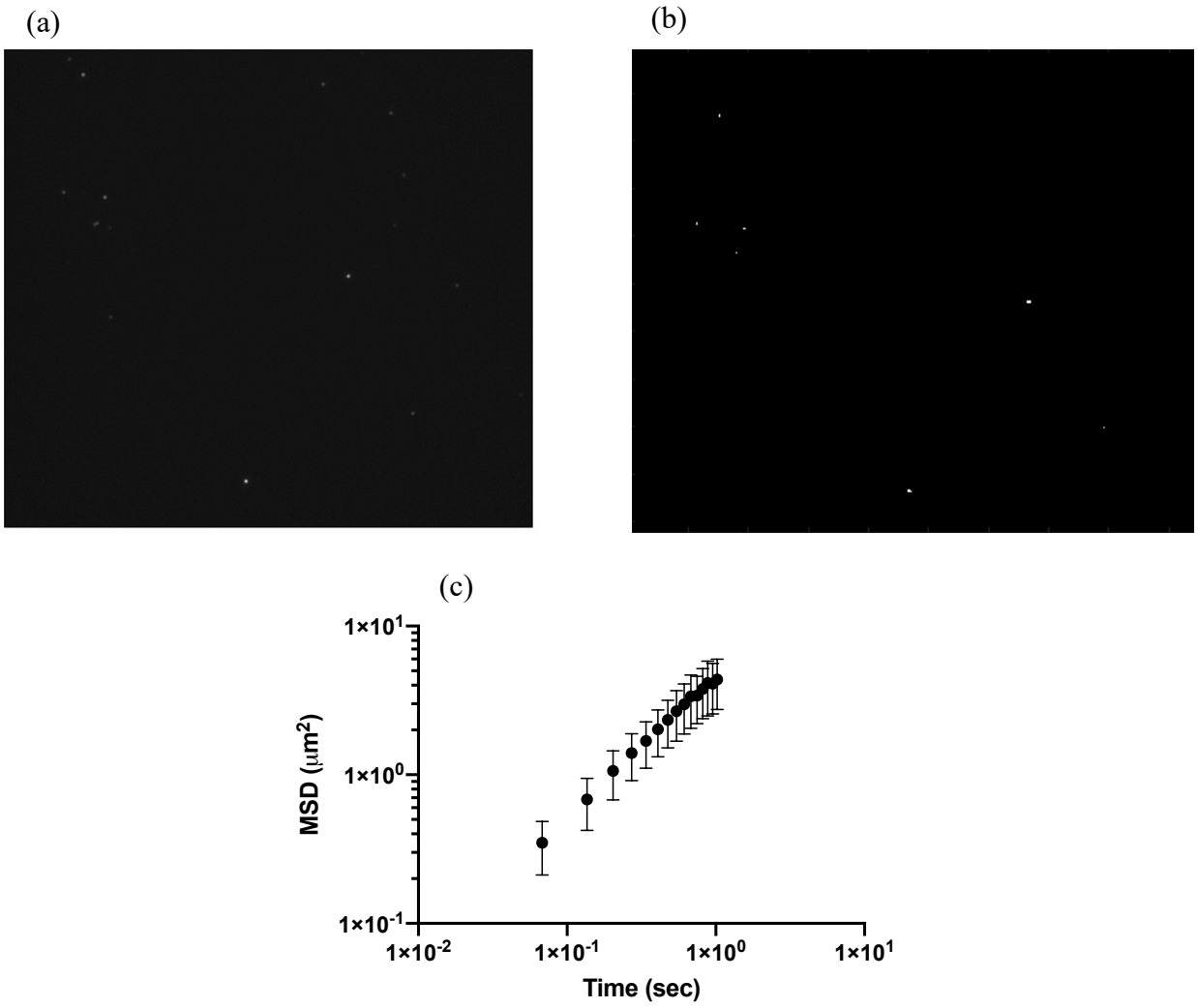

Figure S4. (a) Representative image (in grey-scale) obtained from the time-lapse particle tracking of uncoated polystyrene nanoparticle in PBS. (b) Matlab processed image assuming size 14 pixel (estimated from ImageJ) and cut-off intensity 100. (c) Ensemble-averaged mean square displacement (MSD) of the uncoated polystyrene nanoparticle in PBS as a function of time.

References

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