Tailoring nanoparticle designs to target cancer based on tumor pathophysiology

Edward A. Sykes, Qin Dai, Christopher D. Sarsons, Juan Chen, Jonathan V. Rocheleau, David M. Hwang, Gang Zheng, David T. Cramb, Kristina D. Rinker, and Warren C. W. Chan

Tailoring nanoparticle designs to target cancer based on tumour pathophysiology

Authors: E. A. Sykes[1], Q. Dai[1], C. Sarsons[2], J. Chen[3], J. V. Rochleau[1,4], D. M. Hwang[5], G. Zheng[3], D. Cramb[4], K. D. Rinker[2], W. C. W. Chan[1,7,*]

Affiliations:

[1]Institute of Biomaterials and Biomedical Engineering, University of Toronto.

[2]Biomedical Engineering, University of Calgary

[3]Department of Medical Biophysics, Ontario Cancer Institute, University of Toronto

[4]Toronto General Research Institute, University Health Network

[5]Department of Pathology, University Health Network

[6]Department of Chemistry, University of Calgary

[7]Donnelly Center for Cellular and Biomolecular Research, University of Toronto.

Corresponding Author:

* 164 College Street. Rm 407, Toronto, Ontario, M5S3G9, Canada. warren.chan@utoronto.ca.

Keywords: Nanomedicine, Tumour Size, Nanoparticle Targeting, Tumour Pathophysiology, Extracellular Matrix, Rational Design, Personalized Medicine.

Abstract: Nanoparticles can provide significant improvements in the diagnosis and treatment of cancer. How nanoparticle size, shape, and surface chemistry can affect their accumulation, retention, and penetration in tumours remain heavily investigated as such findings provide guiding principles for engineering optimal nanosystems for tumour targeting. To date, researchers have approached nanoparticle optimization by altering the physico-chemical properties of the nanomaterial. In such attempts, the experimental focus has been on particle design and not the biological system. Here, we varied tumour volume to determine whether cancer pathophysiology can influence tumour accumulation and penetration of different sized nanoparticles. Monte-Carlo simulations were also employed to model the process of nanoparticle accumulation. We discovered that changes in pathophysiology associated with tumour volume can selectively change tumour uptake of nanoparticles of varying size. We further determine that nanoparticle retention within tumours depends on their frequency of interaction with the perivascular extracellular matrix for smaller nanoparticles, while transport of larger nanomaterials is dominated by Brownian motion. These results reveal that nanoparticles can potentially be personalized according to a patient's disease-state to achieve optimal diagnostic and therapeutic outcomes.

Significance Statement: Nanotechnology is a promising approach for improving cancer diagnosis and treatment with reduced side-effects. A key question that has emerged is what is the ideal nanoparticle size, shape, or surface chemistry for targeting tumours? Here, we show that tumour pathophysiology and volume can significantly impact nanoparticle targeting. This presents a paradigm shift in nanomedicine away from identification of a universal nanoparticle design for cancer detection and treatment. Rather, our results suggest that future clinicians will

be capable of using tumour characteristics to tailor nanoparticles according to the patient. This concept of "personalized nanomedicine" was tested for detection of prostate tumours and successfully demonstrated to improve nanoparticle targeting by over 50%.

\body

INTRODUCTION

Nanotechnology remains an emerging and important research discipline for detecting and treating cancer. Unlike small molecules, nanomaterials such as gold nanoparticles, quantum dots, polymeric nanocapsules, and micelles may provide a means of tailoring cancer delivery vehicles for a specific tumour size, state, or type. Nanomaterials can be engineered with different sizes, shapes, and surface chemistries and more recently, they can be assembled into hierarchical nanosystems (1). Nanomaterials can also be engineered with unique properties such as emission of light for fluorescence detection (2), magnetism for magnetic resonance imaging (3, 4), and thermal emission for ablation of tumour cells (5). Despite the potential of nanomaterials, typically less than 5% of an administered dose reaches the tumour compartment (6) due to poor retention within the tumour space and non-specific uptake by the skin (7), spleen, and liver(8–10). Refinements to the size, shape, and surface chemistry of nanomaterials have improved their blood half-lives (11, 12) and interactions with cancer cells (13–15). Unfortunately, targeting efficiency remains stagnated by adherence to the pharmacological ideology that chemicals can be designed to “universally” detect and treat tumours independent of type or stage of cancer progression by varying therapeutic doses. Tumour growth leads to physiological changes in their tissue composition (cell density, vascularity, necrosis, and stroma). If nanoparticles could be tailored according to the physiological state of each tumour, cancer detection and treatment may

be drastically improved. However, investigations into the effect of tumour pathophysiology on nanoparticle accumulation and kinetics have been limited.

Fundamental analysis of tumour pathophysiology has identified unique cellular and structural properties associated with various stages of cancer progression. We currently understand that the increasing vascular tortuosity, inhomogeneity and restricted blood flow (and subsequent low blood pressure) associated with tumour growth prevents chemotherapeutic agents from reaching their target. This impairment of drug delivery may lead to poor therapeutic efficacy and cancer recurrence (16, 17). As we learn more about the cellular, vascular, and compositional characteristics of tumours, it is increasingly evident that tailoring drug delivery vehicles to the physiological state of a tumour may be instrumental to improving treatment of this disease (18, 19). However, enabling clinicians to personalize patient care will require a deeper understanding of how to detect and exploit tumour anatomy and pathophysiology for precise delivery and release of medicinal agents at the tumour site.

Here, we determine whether the delivery of spherical gold nanoparticles (AuNPs) can be affected by changes in tumour volume - a surrogate of cancer progression. Specifically, we (i) characterize how the physiological structures in the microenvironment of orthotopic MDA-MB-435 tumour xenografts of human breast melanoma mature with increasing tumour volume and (ii) explore how such changes can impact uptake, permeation, and retention of polyethylene glycol (PEG)-coated AuNPs. Understanding these variations will enable clinicians to personalize cancer therapy by catering nano-therapeutic regimens according to tumour characteristics. As a proof of concept, we successfully demonstrate that observable changes in tumour pathophysiology can be used in a decision matrix to rationally select AuNP-designs according to desired function.

RESULTS

Characterization of tumours. Pathophysiological changes associated with tumour volume were studied to identify biological parameters that might impact AuNP targeting. The degree of vascularization, cell density and extracellular matrix (ECM) content of different-sized orthotopic human breast melanoma xenograft tumours derived from MDA-MB-435 cells in CD1 nude athymic mouse models were characterized. These parameters were selected as they have been shown to individually impact nanoparticle uptake rate, accumulation, and retention (20–22). Histological sections stained with CD31 antibodies were used to colourimetrically visualize tumour blood vessels while Movat's Pentachrome staining was performed to highlight nuclei and ECM components such as proteoglycans, mucopolysaccharides, and collagen. Vascular density was calculated by counting the number of vessels per tumour cross-section. We observed that the concentration of blood vessels increased with tumour volume but plateaued at 44 ± 3 blood vessels/mm^2 for tumour volumes exceeding 1.0 cm^3 (fig. 1A). Interestingly, the tumour vasculature was only uniformly distributed in small tumours. Tumour blood vessels became increasingly concentrated near necrotic regions and at the tumour perimeter as tumours enlarged (fig. S1).

Beyond tumour vascularization, the fraction of the tumour composed of proteoglycans and mucopolysaccharides increased at a rate of 4.2 a.u./cm^3 (fig. 1B) while tumour-cell density increased at a rate of 1.70 cells/cm^3 (fig. 1C) with tumour volume. Unstained acellular space also proportionally decreased with tumour growth (fig. 1D). These factors coincided with heightened ECM production at regions surrounding tumour blood vessels and necrotic tissue, while ECM-content in regions of dense tumour tissue became reduced (fig. S2). A closer examination of ECM composition by Picrosirius red staining (fig. 2A) and second harmonic generation (SHG)

imaging (fig. 2B) identified that these regions contained type I collagen whose density and structure evolved with tumour growth. Picrosirius red stained samples spectrally shifted from deep red to pale pink (fig. 2C) while SHG microscopy images decreased in intensity (fig. 2D) as tumours enlarged. The 9% cm^{-3} reduction in Picrosirius red intensity and spectral shift in SHG peak intensity were characteristic of a loss in structural ECM via reduction in collagen fiber thickness and length (23–25).

Together, these results indicate that as tumours mature through growth, their tissue and vasculature become denser and more chaotic. In particular, the ECM appears to remodel during tumour enlargement, thus leading to a more amorphous phenotype. Given that ECM components were observed to encapsulate tumour blood vessels (fig. S3) and are known to biologically function as a basal-support for blood vessels that interfaces with the stroma, changes in ECM may be a primary mediator of nanoparticle entry into the tumour compartment.

Gold nanoparticle model system. Having characterized the evolution of tumour tissues during growth, we sought to determine whether these physiological changes could be used to tune the tumour targeting efficacy of nanoparticles. As tumour uptake is dependent on nanoparticle diameter (12, 26, 27), a library of methoxy-PEG coated AuNPs of varying diameter were designed to examine how tumour growth would affect particle delivery. While clinical trials for AuNPs are limited, AuNPs were selected over more clinically appropriate polymeric nanomaterials as they can be reproducibly and precisely synthesized in a broad range of sub-100 nm sizes. Furthermore, AuNPs provide a non-deformable formulation for testing the effect of core-diameter on tumour uptake, are easily surface modified, and can be quantified in tissues with high sensitivity. A schematic illustrating the AuNP design employed in this study is depicted in fig. S4A.

Spherical AuNPs with core-diameters of 15, 30, 45, 60, and 100 nm (fig. S4B) were synthesized using standard citrate and hydroquinone reduction techniques (28). These sizes were selected to systematically characterize how the tumour microenvironment would impact a broad range of particle diameters. AuNP surfaces were modified with hetero-bifunctional 5 kDa polyethylene glycol (PEG) with methoxy- and sulfhydryl- termini as well as Alexa Fluor 750 labelled 10 kDa sulfhydryl-PEG to respectively stabilize particles for blood transport and to fluorescently track particles *in vivo*. Although it is difficult to use fluorescence as an absolute quantification technique, we have shown previously that it is an accurate modality for monitoring relative changes in nanoparticle biodistribution (26, 29). Surface modifications resulted in AuNPs with a PEG-packing density of 0.3-1.5 ligands/nm^2. At these densities, surface-bound PEG moieties were calculated according to their Flory diameter to be in the brush layer conformation ensuring that the tested nanoparticles were sufficiently passivated (table S1). Surface modifications were also found to increase nanoparticle hydrodynamic diameters by 20 – 40 nm (fig. S4C), and positively shift nanoparticle zeta potentials by 20 – 30 mV (fig. S4D). Particle fluorescence was confirmed by the migration of distinct fluorescent bands during agarose gel electrophoresis (fig. S4E). AuNP-fluorescence was shown to increase proportionally with particle diameter (fig. S5). Fluorescent PEG groups were also confirmed to be stably bound to particle surfaces as the rate of desorption in the presence of serum remained below 0.2 PEG/hour (fig. S5D). *In vivo* pharmacokinetics of our functionalized AuNPs was also characterized by analysis of blood plasma at 0, 2, 4, 8, and 24 hours post-tail vein injection (HPI) in non-tumour bearing CD1 nude athymic mice. Inductively coupled plasma atomic emission spectroscopy (ICP-AES) analysis of blood samples revealed that the blood half-lives of our

AuNPs ranged from 2 to 10 hours. A complete characterization of our formulations is presented in table S2.

Analysis of nanoparticle accumulation in tumours. AuNP accumulation was evaluated *via* tail-vein injection of formulations into CD1 nude athymic mice bearing orthotopic MDA-MB-435 human breast melanoma tumours. Tumours volumes evaluated in this study ranged from 0.05 – 3.00 cm^3. AuNP delivery to the different sized tumours was fluorescently profiled in mice to assess tumour accumulation kinetics and to measure total AuNP exposure. Fluorescent tracking was achieved by whole animal imaging using a Carestream *In Vivo Imaging System* at time points ranging from 0 – 24 HPI.

Total area under the curve (AUC) was calculated from the kinetic curves seen in fig. S6 as a metric for AuNP accumulation within the tumour. Overall, AUC values increased with tumour volume (fig. 3A). Accumulation for 15, 30, and 45 nm AuNPs steadily increased with tumour volume from 490±70 to 720±30% ID·h, 280±50 to 750±10 ID·h, and 480±70 to 960±100%ID·h respectively. Changes in accumulation of larger formulations occurred as step increases at discrete tumour volumes. Uptake of 60 nm formulations was ~1.5 times higher once tumours exceeded 2.2 cm^3 while 100 nm particles exhibited a ~4.6 times increase in accumulation for volumes 0.5 cm^3 and larger. These trends were confirmed by ICP-AES measurements of gold content in tumours at 24 HPI (fig. 3B). The ICP-AES results indicated that by 24 HPI tumour uptake of 15 and 30 nm particles were consistently higher than all other formulations and steadily increased from 0.39±0.04 to 0.99±0.18%ID and 0.28±0.03 to 0.90±0.18%ID respectively (two-way ANOVA, $p = 0.05$), while larger particles such as 60 nm trended higher (though statistically not significant) from 0.18±0.02 to 0.26±0.12%ID as tumour volumes were enlarged.

In combination with our histological observations, these results suggest that the higher porosity of the ECM increasingly accommodates the entry of larger nanoparticles at later stages of tumour growth. This implies that a minimum tumour size must be reached to support entry of each AuNP diameter. An AuNP accumulation threshold of 500% was selected to illustrate this point (fig. 3A). This threshold was defined as the mean AUC of 15 nm AuNPs in sub 0.5 cm^3 tumours as particles in this size range would experience the least steric hindrance. AUC values for each AuNP diameter were statistically compared to the threshold (two-way ANOVA, $p = 0.05$). 15 nm AuNPs reached this accumulation threshold at tumour volumes of 0.5 cm^3 and larger, while 30 nm nanoparticles achieved a similar trend at threshold of 0.5-1.0 cm^3 and above. Similarly, 45 nm formulations attained statistically higher accumulation at tumour volumes above 1.0 cm^3 and 60 nm AuNPs exceeded this threshold (though statistically insignificant) when tumour volumes were beyond 2.2 cm^3. 100 nm particles never reached the defined threshold accumulation at any of the tumour volumes tested. It has been shown that AuNPs greater than 100 nm in diameter sequester near tumour blood vessels and do no penetrate into MDA-MB-435 tumours (26, 27). Hence, the difference in the accumulation pattern of 100 nm AuNPs over the other tested formulations was attributed to steric hindrance (possibly due to obstruction of ECM pores).

Nanoparticle kinetics within the different sized tumours. Kinetics of AuNP delivery to tumours were analyzed in an effort to explain the dependence between accumulation and tumour volume. Tumour uptake rates were calculated by taking the instantaneous slope at 3 HPI of the AuNP accumulation profiles presented in fig. S6. We observed that the speed of AuNP accumulation (fig. 3C) was largely insensitive to changes in tumour volume (two-way ANOVA, $p = 0.05$). 15, 60 and 100-nm AuNPs maintained tumour entry rates of 4.2 ± 0.6, 3.2 ± 0.9, and 2.9 ± 0.8

%ID·hour^{-1} as tumours grew up to 1.0 cm^3. Particles with 30 nm and 45 nm diameters were the exception as their rate of uptake steadily rose from 2.3 ± 0.3 to 5.7 ± 0.9 %ID·hour^{-1} and 2.8 ± 0.9 to 7 ± 1 %ID·hour^{-1} respectively, as tumours grew beyond 0.5 cm^3. Although the rate of delivery did not statistically vary with growth, AuNP entry into the tumour compartment trended higher as tumours increased in size. The 15, 30, and 45 nm AuNPs also consistently accumulated in tumours ~1.2-1.7 times faster than our 60 and 100 nm formulations. However, these differences became less apparent as tumour volumes increased. These results further reinforce the relationship between ECM porosity and particle size whereby smaller pores restrict larger nanoparticles from deep tumour infiltration and conversely become washed out of the tumour at a faster rate than smaller nanomaterials.

Since it is difficult to probe nanoparticle transport through ECM in animal models, we developed an *in vitro* system to measure diffusion of AuNPs into a hydrogel to mimic the effects of collagen structure on the transport of nanoparticles into the tumour (fig. 4A). Although this *in vitro* model only evaluates diffusion through a collagen matrix independent of fluid flow or cellular interactions, it provides a means to determine how the velocity of transport and quantity of AuNPs within tumours are dictated by the perivascular stroma upon initial AuNP entry. Self-assembled hydrogels composed of either 2.5 or 4.0 mg/mL of type I collagen were used to mimic stromal changes caused by tumour growth. Type I collagen was selected as a stromal-phantom as it is the primary component of the tumour-blood vessel interface (30, 31). Entry of AuNPs from a fluid reservoir into the hydrogel was kinetically monitored by AuNP fluorescence using scanning confocal microscopy at different time-points over 900 minutes. Overall, AuNP transport into the collagen gel occurred in two phases: (i) rapid concentration at the periphery of

the hydrogel and (ii) gradual movement from the concentrated zone to deeper regions of the matrix (fig. 4B).

The quantity of hydrogel-infiltrating AuNPs plateaued within 120-240 min post-exposure for all formulations greater than 45 and 15 nm for our 2.5 and 4.0 mg/mL collagen hydrogels respectively (fig. S7A). The AuNP diffusion-front also plateaued by 480 minutes post-exposure for all particle diameters independent of collagen density (fig. S7B). Rather, AuNP penetration into the hydrogel at later time points occurred by diffusing away from the concentrated zone into the surrounding gel (seen in fig. S9 as a broadening of the diffusion-front). This penetration was dictated by particle diameter. 45 nm AuNPs achieved the highest permeation at 17.0 ± 2.0 µm and 13.2 ± 0.4 µm, whilst 100 nm AuNPs exhibited the poorest penetration at 8.0 ± 1.0 µm and 5.4 ± 0.6 µm for collagen densities of 2.5 and 4.0 mg/mL respectively (fig. 4C). Although AuNP permeation appeared to decrease with collagen concentration, differences were not statistically significant (two-way ANOVA $p > 0.05$). These trends were consistent with our tumour permeation results seen at 24 HPI where AuNP infiltration did not vary with tumour volume (fig. 4D). Particle permeation also did not change statistically between the tumour periphery, regions neighboring necrotic zones, or within the core of the tumour tissue. Despite the lower collagen density, diffusion of 15 nm AuNPs into the 2.5 mg/mL collagen gels was unexpectedly 2.0 and 1.3 times lower than our 45 nm formulation *in vitro* and *in vivo* respectively. These differences in diffusion were similar to previous studies (26, 27) and were attributed to the speed of AuNP uptake by and expulsion from the collagen matrix (fig. 4E &F). Uptake and expulsion of 15 nm formulations were respectively 14% slower and 51% faster in 2.5 mg/mL gels than the denser matrix. This leads to a lower AuNP concentration within the collagen gel and accordingly, slower particle diffusion. Alternatively, as the uptake rate of AuNPs exceeding 45 nm does not

vary with collagen density (two-way ANOVA, $p = 0.05$), their slower depletion from the collagen matrix allows for greater nanoparticle retention and consequently greater infiltration distances.

Computational modelling of nanoparticle diffusion through porous matrices. To help understand how nanoparticles interact with the collagen matrix, Monte-Carlo numerical simulations of AuNP diffusion through collagen matrices were conducted. Two-dimensional models were used to examine the frequency of AuNP collisions with collagen-fibers within square pores of the hydrogel matrix. The frequency of such collisions can cause an AuNP's path to deviate. Three-dimensional simulations were also conducted to compare AuNP permeation capacity through stroma of different collagen densities. These computational models were conducted in Matlab using custom algorithms to simulate AuNP interaction and diffusion within collagen matrices. These simulations followed similar strategies employed by Stylianopoulos et al. (32). AuNP motility was modelled as step-wise random walk obeying Einstein-Stokes diffusion (Equation 2) while particle-fiber interactions were modelled as elastic collisions. Figure 5A provides an illustration delineating the path of AuNP motion within a collagen pore. Obeying Brownian motion, AuNPs move randomly and can collide with collagen fibers. For our three-dimensional simulations, collagen fibers were approximated as cylinders with radii between 0.05 – 0.50 µm. Representative images of the collagen matrices of varying collagen density simulated in Matlab have been presented in fig. 5B. The modeled radii were chosen according to measured thicknesses from scanning electron microscopy images of our 2.5 and 4.0 mg/mL collagen hydrogels (fig. S9). A detailed summary of our model and its underlying assumptions can be found in the methods section.

AuNP movement in our two-dimensional models for 1000 particle replicates was simulated in 0.005, 0.020, 0.108 and 0.640 μm^2 square stromal-pores for 10000 steps at 0.1 second intervals. Our simulations determined that AuNP-fiber collision rates increased with reducing pore size and decreasing AuNP diameter (fig. 5C). 15 nm AuNPs achieved the highest frequency of interaction with collagen fibers at rates between 0.038 - 0.023 collisions/second (cps) while 100 nm formulations ranged from 0.016 – 0.001 cps for pore sizes between 0.005 – 0.640 μm^2. Interestingly, collision rates for 15, 45, and 60 nm AuNPs were statistically similar for 0.005 μm^2 pores (ANOVA $p = 0.05$) but became increasingly dissimilar as pores enlarged. These simulations suggest that impact of particle size on Brownian motion is a primary mediator of AuNP motility within the hydrogel over its frequency of collision with the ECM. This suggests that the greater that nanoparticles interact with collagen, the longer they will be retained within the tumour.

Expanding on these results, AuNP diffusion was also modelled in three-dimensions to compare how AuNP diameter and collagen density might impact stromal accumulation and infiltration. Stromal-ECM of increasing collagen density was modelled computationally as 27000 μm^3 cubes containing anisotropically oriented collagen fibers. The number of fibers were chosen to achieve collagen volume fractions (8.72 – 87.20%) reflective of conditions found within tumours (33, 34). Diffusion distance for 500 AuNP-replicates was tracked for 5000 discrete steps at 1 second intervals. Our simulations indicate that diffusion rates changed with AuNP-diameter but did not change with collagen density (fig. 5C). 15 nm AuNPs exhibited the greatest mobility at 1.95 ± 0.03 nm/s in the simulated hydrogels while 45, 60, and 100 nm particles diffused at rates of 0.78 ± 0.01, 0.60 ± 0.01, and 0.36 ± 0.01 nm/s respectively. These

findings support our AuNP permeation observations from histological tumour sections whereby AuNP diffusion away from blood vessels (fig. S10) did not vary with tumour volume (fig. 5D).

Together, these 2D and 3D models elucidate how the stromal matrix is implicated in particle permeation. Although these simplified 2D and 3D models ignore the effect of fluid flow, oncotic pressure, and inelastic collagen-AuNP interactions, they provide a mechanism for our *in vitro* and *in vivo* permeation observations. They suggest that AuNP permeation is the balance between the effects of particle size on Brownian motion and the frequency of particle collision with the ECM. The increased mobility of smaller AuNPs afforded greater diffusion but was also inhibitory due to the higher frequency of collision with the ECM. Conversely, AuNPs of larger diameters exhibited slower motion but also a lower propensity to interact with the stroma. The volume fractions tested and simulated in this study equate to ECM pore sizes ranging from 0.45 – 1.74 μm. As these pores exceed the size of our AuNPs, differences in diffusivity associated with collagen density would be negligible for all tested particle diameters. Extended further, these computational findings demonstrate that AuNP transport within the tumour can be distorted through collisions with ECM fibers. This can limit retention within the tumour compartment if AuNP volume approaches the porosity of the stromal ECM. It is likely that as nanoparticles move through the tumour matrix, they interact with ECM fibers circumferential to pores or become trapped in zones of varying size.

Nanoparticle selection according to tumour maturity. Given the complex dependence of tumour-AuNP uptake on both particle size and tumour pathophysiology, we asked whether there was a means to rationally select AuNP formulations according to tumour volume. In our proof-of-concept work, we evaluated whether a decision matrix could be used to select nanomaterials for either tumour detection (diagnostic) or drug delivery (treatment). AuNP formulations with

315 rapid delivery and high tumour contrast were defined as effective probes for delineating tumours
316 while AuNPs capable of high tumour retention and homogeneous tissue distribution were
317 anticipated to fare well as drug delivery vehicles.

318 Relative measurements of AuNP-fluorescence *in vitro* and *in vivo* (fig. S5A & S5B) were
319 used as an estimate of tumour contrast achievable by each formulation. Surface area-to-volume
320 ratios were also calculated to approximate the drug-loading capacity of each AuNP size (fig.
321 S7B). These parameters in conjunction with tumour accumulation, uptake rate, and penetration
322 capacity were ranked from best [4] to worst [1] for each AuNP diameter. Each parameter was
323 also given a multiplier according to its importance to a given AuNP-function. The weighted sum
324 of these rankings was then calculated for each AuNP design for the different tumour size ranges.
325 Equation 1 is a summary of the scoring scheme where μ is the importance multiplier, β
326 represents the ranking factor, and *i* denotes the ranked AuNP parameters. Figure 6B highlights
327 these parameters and the associated values used to calculate the scores found in our decision
328 matrices (fig. 6A-6B).

329 $$Score_{AuNP}|_{Tumour} = \sum_i \mu_i \cdot \beta_i \qquad (1)$$

330 Overall, smaller (< 45 nm) AuNPs were favored for both diagnostic and therapeutic applications
331 across all tumour sizes. Diameters in the 100 nm range were consistently predicted as poor
332 candidates for either application while 15 nm and 45 nm particles were both expected to be
333 useful for detection and treatment of large (> 1.0cm^3) tumours. AuNPs in the 60 nm range were
334 the exception to these trends as they were predicted to be better for detection of small, early-
335 stage tumours (<0.5 cm^3). This was empirically attributed to the statistical similarity in AUC
336 values for particles with diameters between 15-60 nm (fig. 3A) as well as the higher tumour
337 contrast seen for 60 nm particles (fig. S5) in the 0.0-0.5 cm^3 range. As macrophage uptake of

nanoparticles increases with particle diameter (35), the enhanced utility of 60 nm AuNPs may also be related to changes in phagocytic capacity of tumour associated macrophages (36) as their phenotypes evolve during tumour progression (37).

These results imply that passively targeted AuNPs with smaller diameters would be more applicable for detection and drug delivery when tumour size is unknown. However, 45 nm AuNPs may be the more effective vehicle for later-staged tumours as their larger surface area to volume ratio (fig. S5C) theoretically allows for 900% greater drug loading than 15 nm particles with merely a drop to tumour accumulation by less than 57.3%. Although these findings are specific to passively targeted AuNPs with further research and amalgamation with the existing wealth of information on nanoparticle-tumour targeting, the proposed decision matrix schema can be generalized to provide a systematic method for assessing other particle types. A flow chart detailing a potential means of implementing this strategy is outlined in fig. 6C.

Validation of the decision matrix for personalized targeting of prostate tumours. Towards validating our results, we evaluated whether our formulated decision matrices could be used to predict the ideal AuNP design for other tumour models. A blinded study was conducted in CD1 nude athymic mice bearing orthotopic PC3 prostate human tumours to verify whether our tumour-size dependent predictions were accurate. 15 and 100 nm AuNPs were tail-vein injected into tumour bearing mice to evaluate AuNP efficacy for tumour detection and accumulation. Both particle designs were effective at delineating the location of the tumour (fig. 7A) but at varying efficacies. Tumour detection speed and contrast for 15 nm AuNPs were respectively 53.7% and 50.8% higher than 100 nm particles. 15 nm achieved greater tumour accumulation than 100 nm designs and trended higher with increasing tumour size (fig. 7C-7E). These findings

were consistent with our decision matrix alluding to the potential of our system for use on other particle formulations and tumour types.

DISCUSSION

Given the observed limitations of AuNP accumulation in tumours, it is clear that careful design of nanomaterials is necessary to achieve optimal tumour delivery. It is currently known that manipulating the diameter, shape, and surface chemistry of a nanomaterial can yield particles that minimally interact with the hepatic and renal clearance mechanisms of the body (38). However, the design of AuNPs must also be finely balanced with function to achieve optimal delivery of payloads and signal intensities. Unfortunately, optimization of the synthetic identity of nanomaterials has reached an impasse whereby tumour targeting efficiency remains stagnated at 5% (6, 39). In our work, we have alternatively approached tumour delivery from the biological perspective by characterizing the unique physiological changes that occur during tumour growth to tailor nanoparticles according to the state of disease progression.

We determined that for MDA-MB-435 orthotopic human tumour xenografts, malignant tissues become more disordered as they increase in volume. Starting from homogeneously-vascularized tissues with minimal necrotic space, tumours transition towards higher cell densities with vasculature that concentrates at sparsely distributed regions. This disproportionate vascularization coincides with an increase in necrotic tissue and expression of collagen, and other ECM components that surround tumour blood vessels. Type I collagen in the tumour ECM was found to convert from long filamentous fibers to shorter and more amorphous structures as tumours increase in volume. Through use of an *in vitro* collagen hydrogel model, we rationalized that these structural changes in the ECM are a primary mediator of passive tumour delivery of spherical AuNPs. This collagenous basal membrane acts as a “sponge” for extravasating AuNPs

that delays particle infiltration. The densely packed ECM of early-stage tumours appears to sterically restrict AuNP entry based on particle diameter while in larger tumours, the more porous and less rigid structure of type I collagen facilitates entry of larger AuNPs and enhances accommodation of smaller particles by the stroma. As AuNP infiltration depth did not change with tumour size *in vivo* nor with variations to collagen density *in vitro,* bulk tumour accumulation of particles likely depends (i) on the capacity of ECM to take up AuNPs and (ii) on the number of blood vessels available for AuNP entry for a given tumour volume.

These tumour growth-associated changes highlight physiological parameters that are exploitable for selection of AuNPs according to tumour volume. The reduction of available interstitial volume and enhanced porosity of stroma caused by tumour growth hinder permeation of AuNPs but allow for higher AuNP extravasation into the tumour space. This suggests that although large AuNPs become more effective when tumours mature, this improvement in accumulation comes at the expense of deep tissue permeation. Early prognosis and treatment of cancer is associated with increased patient survival (40–42). Unfortunately, smaller AuNPs which are best suited to target low-volume tumours may be less effective drug delivery vehicles as their payloads maybe smaller than their counterparts. This illustrates the dichotomy of AuNP selection, as a trade-off must be made between the intended function of a nanomaterial and optimal tumour delivery.

Towards personalized medicine, a simplified decision matrix was developed to illustrate a means of personalizing the selection of AuNPs according to tumour stage and desired AuNP function. Our proof of concept decision matrix facilitates the personalization of a nanomaterial according to the patient by providing an unbiased score of how well a formulation might fare based on tumour volume and the AuNP's design parameters: tumour signal (fluorescence),

406 accumulation, uptake rate, and permeation. Figure 6B presents a flow chart illustrating how such
407 a decision matrix might be used clinically to select nano-therapeutic regiments.

408 Simulations established that for MDA-MB-435 tumours, passively targeted AuNPs with
409 60 nm diameters provide the best contrast for detecting early stages of tumour growth, and sites
410 of metastasis. Alternatively, particles in the 15 – 45 nm range appear to be more effective for
411 diagnostics as tumours increase in size or in situations where tumour maturity and phenotype are
412 unknown. For therapeutic regimens, our work also identifies that AuNPs with diameters between
413 15 – 45 nm are best employed for tumours exceeding 1.0 cm^3 as their permeation distance
414 exceed 60 – 100 nm AuNPs despite having lower loading capacities. These results imply that
415 AuNPs must be rationally designed according to the intended function. Formulations optimized
416 for diagnostic applications may not necessarily be effective designs for drug delivery or vice
417 versa. Although we have shown that our AuNP-tumour size trends were also valid for a PC3
418 prostate tumour model, our results may not necessarily be generalizable to all tumour types as
419 the decision matrix presented here was constructed from a single tumour type and nanoparticle
420 design. However, as our proposed decision matrix utilizes phenotypic parameters that are
421 common to malignant tissues allowing the proposed strategy to be easily adopted by pathologists
422 and researchers. With a concerted effort amongst researchers to elucidate how different
423 nanoparticle schemes as well as the different micro-architectures of different tumour models and
424 host species can impact nanoparticle entry and retention within tumours, a generalized decision
425 matrix may be realized. Production of this large database may allow future clinicians to utilize
426 standard magnetic resonance, computer tomographic, histological imaging techniques to
427 landmark and approximate the size of a tumour so as to synthesize nanoparticle based treatments
428 that are catered specifically to the patient.

CONCLUSIONS:

To improve cancer detection and therapy, researchers are now investigating how the physicochemical properties of a nanomaterial can mediate nanoparticle transport and function. Although it is clear that the synthetic properties of the nanoparticle are critical to their biological interactions, how the physiological characteristics of the tumour can impact nanoparticle fate remains largely unexplored. Here, we show that tumour biology is equally important as nanoparticle size in dictating nanoparticle targeting efficacy. We further show that a thorough assessment of tumour composition can be used to develop a simple algorithm for rational selection of AuNPs according to cancer stage. Implementation of nanomaterials in tandem with radiological imaging and tissue biopsies may be clinically useful to optimally detect nascent tumours and personalize therapeutic regimens. However, realization of this personalized approach to cancer nanomedicine will require a greater understanding of the physical changes in tumour microenvironment associated with cancer progression and its implications on nanoparticle function.

The conclusions presented here have been formulated with passively targeted AuNPs using an orthotopic MDA-MB-435 tumour model. Although we successfully demonstrate that our proposed decision matrix can predict AuNP targeting efficacy for orthotopic prostate tumours, ascertaining how tumour growth can affect malignant tissues in other tumour models remains critical to ensure that animal and nano-based research can be translated to humans. It would also be prudent to study how other nanoparticle types and targeting schemes may change nanoparticle interactions with the host and tumour microenvironment. For example, analysis of how tumour pathophysiology influences active targeting may help to explain why the decoration of bio-recognition molecules on nanoparticle surfaces appear to only enhance tumour targeting

for nanoparticles within the 60 nm range (26). Further investigation on such topics will broaden our understanding of nano-bio interactions and allow for the development of a fundamental framework for design of cancer-centric nanomaterials. Nevertheless, our results illustrate that tumour maturity is a critical parameter that both impacts the fate of a nanomaterial and can be exploited to rationally design better diagnostic probes and therapeutic vehicles in the future.

METHODS

Tumour accumulation measurements. Efficiency of AuNP delivery to tumours was measured by ICP-AES. Tumours were harvested at 24 HPI and digested in 1 mL of *aqua regia* (1:3 v/v nitric acid to hydrochloric acid) supplemented with 1 µg/mL yttrium for 2 hours at 70˚C. Yttrium was used as an internal reference to account for sample loss during the digestion and purification process. Post-digestion, acidic solutions were diluted with 2 mL of double distilled water and filtered through 0.22 µm PVDF membranes to remove un-digested tissue. Volumes of the digested samples were then adjusted to achieve a final volume of 4 mL via addition of double distilled water. Gold and yttrium contents in each sample were measured using a Perkin-Elmer Optima 3000. AuNP accumulation in tumours at 24 HPI was determined by normalizing measured gold concentrations to yttrium content and tumour mass.

Analysis of nanoparticle infiltration into collagen matrices. Synthesized nanoparticles were tested *in vitro* for their permeation capacity through type I collagen hydrogels. Self-assembled hydrogels were first prepared by mixing pre-solubilized rat tail type I collagen on ice with 10x phosphate buffered saline, and 1 M sodium bicarbonate at an 8:1:1 volumetric ratio followed by dilution with double distilled water to achieve final collagen concentrations of 2.5 and 4.0 mg/mL. Collagen solutions were then placed into gel moulds and allowed to self-assemble at

37˚C for 3 hours. Post-polymerization, hydrogels were equilibrated in double distilled water for 2 hours followed by immediate water-exchange and introduction of AuNPs. AuNP infiltration into hydrogels was monitored every 30 minutes for 15 hours via laser scanning confocal microscopy using an Olympus Fluoview FV1000. A trans-illumination lamp was used to determine the collagen edge while differential interference contrast (DIC) and fluorescence were invoked to profile AuNP distribution within the hydrogel. AuNP permeation was profiled along the length of the collagen hydrogel by analyzing confocal images of AuNP fluorescence in ImageJ. Fluorescent intensity profiles were then placed into Graphpad Prism to calculate total AuNP uptake and track the mean AuNP infiltration distances. Calculated values were used to determine AuNP accumulation rates for the different hydrogel densities by taking the slope of the linear regression curves seen in fig. S12A.

Analysis of nanoparticle expulsion from collagen matrices. Collagen hydrogels were constructed using a similar pH-based self-assembly process as mentioned above. Prior to gelation, AuNPs equivalent to a total surface area of 30 cm^2 were thoroughly mixed with hydrogel solutions on ice. AuNP-collagen mixtures were then allowed to set overnight at 37˚C, rinsed with phosphate buffered saline, and suspended in 1 mL of phosphate buffered saline. At 0, 1, 2, 3, 5, 6, 8, and 24 hours the phosphate buffered saline suspension solution was sampled (90 µL) to track AuNP expulsion from the hydrogels. AuNP quantity in sample solutions was approximated by measurement of sample fluorescence in 384 fluorescent well plates (Nunc 384-well optical well plates) using a Carestream Multispectral MS Fx Pro in vivo imager (ex/em: 750/830 nm) at an exposure time of 10 minutes. Fluorescent images were analyzed by

densitometry in ImageJ. AuNP expulsion rates were obtained by taking the slope of the linear regression curves seen in fig. S12B.

Simulation of nanoparticle diffusion in collagen matrices. Two- and three-dimensional stochastic models of AuNP movement in collagen matrices were programmed and simulated in Matlab. Two-dimensional models were used to study how AuNP diameter and differences in the available area fraction of collagen matrices would affect the frequency of AuNP-collagen collisions. Three-dimensional simulations were conducted to investigate the impact of collagen density on AuNP diffusion distance. For both models, AuNP movement was taken as discrete random walk steps obeying Einstein-Stokes Brownian motion as dictated by equation 2 where K_B, η, T, and r denote the Boltzmann constant, solvent viscosity, temperature in kelvins, and AuNP radii respectively.

$$D = \frac{\kappa_B T}{6\pi\eta r} \quad (2)$$

Particle movement was approximated as the mean square displacement according to Fick's second law (equation 3). Where δ and dt were taken as the discrete distance and time interval between steps.

$$\delta = \sqrt{2Ddt} \quad (3)$$

AuNPs were approximated as circles and spheres for two- and three-dimensions respectively. AuNP-collagen fiber collisions were assumed to be elastic with collagen fibers approximated as immobile cylinders. Simulations were also conducted in the limit of dilute AuNP concentrations where AuNP-AuNP collisions could be neglected and particles could be independently tracked.

For the two-dimensional simulations, collagen matrices of differing available area fractions were approximated as square pores of varying size. Pore sizes were selected based on empirically measured spaces between collagen fibers seen in scanning electron microscopy images of collagen hydrogels of varying concentration (fig. S9). Images were imported into ImageJ and thresholded to differentiate collagen fibers from pores. The size of each pore was measured by calculating the rolling ball radius of each pore using ImageJ's built-in algorithm. Initial AuNP positions were randomized within the collagen pores and were permitted to move stochastically within the square. Upon AuNP movement beyond the dimensions of the pore, collision events were counted and AuNP trajectories were elastically reflected. Particles were tracked for 10000 steps at 0.1 second intervals. AuNP-collagen collisions were tallied for each condition.

To simulate three-dimensional collagen matrices composed of 100, 300, 600, and 1000 cylinders ranging in length from 0-30 µm and radii ranging from 0.05-0.50 µm were randomly distributed and oriented within 30 x 30 x 30 µm cubes to mimic hydrogels with collagen volume fractions between 8.72 – 87.20%. Collagen volume fractions were determined by calculating the ratio of volume occupied by collagen fibers (approximated as cylinders) versus the total region of interest (30 x 30 x 30 µm cube). Volume fractions were equated to empirical collagen pore sizes by taking a 2D projection of the generated tissue followed by calculation using the same ImageJ process as mentioned for our 2D simulations. For particle motility simulations, AuNPs were randomly placed in our 3D matrices, allowed to move freely within the confines of the cube, and reflect off collagen fibers. The direction of AuNP motion was randomized with each step. AuNPs were tracked for 5000 steps at 0.5 second intervals. Displacement between start and end points were measured to determine AuNP diffusion distances.

Statistical analysis. All statistical analysis comparing between groups were performed using one-way ANOVA (one variable per group) and two-way ANOVA (two variables per group) in GraphPad Prism.

Supplementary information. Please see supporting information section for detailed materials list, gold nanoparticle synthesis and characterization, tumour induction, nanoparticle administration, tumour histology analysis, and whole animal imaging.

References and Notes:

Acknowledgments: We would like to thank the Natural Sciences and Engineering Research council (RGPIN288231) and the Canadian Institute of Health Research (MOP130143, COP126588) and Canadian Research Chair (950-223824) for grant funds. E.A.S. and C.S. would also like to acknowledge the Natural Sciences and Engineering Research council for their fellowships. C.S. would like to acknowledge Alberta Innovates Technology Futures for his scholarship as well.

Funding: Include all funding sources here, including grant numbers and funding agencies.

Author contributions: E.A.S. and W.C.W.C. conceived the project and wrote the paper. E.A.S., Q.D., and C.S. conducted the experiments. D.C. and K.D.R. helped designed the collagen experiments. J.R. helped in the setup of simultaneous SHG and fluorescence imaging. J.C. and G.Z. produced the prostate cancer mouse models used in this study. D.H. conducted the pathology analysis. All authors edited the manuscript.

559 **REFERENCES:**

560 1. Chou LYT, Zagorovsky K, Chan WCW (2014) DNA assembly of nanoparticle
561 superstructures for controlled biological delivery and elimination. *Nat Nanotechnol*
562 9(2):148–55.

563 2. He X, Wang K, Cheng Z (2010) In vivo near-infrared fluorescence imaging of cancer with
564 nanoparticle-based probes. *Wiley Interdiscip Rev Nanomedicine Nanobiotechnology*
565 2(4):349–366.

566 3. Nam T, et al. (2010) Tumor targeting chitosan nanoparticles for dual-modality optical/MR
567 cancer imaging. *Bioconjug Chem* 21(4):578–82.

568 4. Yang J, et al. (2007) Multifunctional magneto-polymeric nanohybrids for targeted
569 detection and synergistic therapeutic effects on breast cancer. *Angew Chem Int Ed Engl*
570 46(46):8836–9.

571 5. Cherukuri P, Glazer ES, Curley SA (2010) Targeted hyperthermia using metal
572 nanoparticles. *Adv Drug Deliv Rev* 62(3):339–45.

573 6. Bae YH, Park K (2011) Targeted drug delivery to tumors: myths, reality and possibility. *J*
574 *Control Release* 153(3):198–205.

575 7. Sykes EA, Dai Q, Tsoi KM, Hwang DM, Chan WCW (2014) Nanoparticle exposure in
576 animals can be visualized in the skin and analysed via skin biopsy. *Nat Commun* 5:3796.

577 8. De Jong WH, et al. (2008) Particle size-dependent organ distribution of gold nanoparticles
578 after intravenous administration. *Biomaterials* 29(12):1912–1919.

579 9. Cole AJ, David AE, Wang J, Galbán CJ, Yang VC (2011) Magnetic brain tumor targeting
580 and biodistribution of long-circulating PEG-modified, cross-linked starch-coated iron
581 oxide nanoparticles. *Biomaterials* 32(26):6291–301.

582 10. Kumar R, et al. (2010) In vivo biodistribution and clearance studies using multimodal
583 organically modified silica nanoparticles. *ACS Nano* 4(2):699–708.

584 11. Moghimi SM, Hunter a C, Murray JC (2001) Long-circulating and target-specific
585 nanoparticles: theory to practice. *Pharmacol Rev* 53(2):283–318.

586 12. Yaehne K, et al. (2013) Nanoparticle accumulation in angiogenic tissues: towards
587 predictable pharmacokinetics. *Small* 9(18):3118–27.

588 13. Huang X, Teng X, Chen D, Tang F, He J (2010) The effect of the shape of mesoporous
589 silica nanoparticles on cellular uptake and cell function. *Biomaterials* 31(3):438–48.

14. Hauck TS, Ghazani AA, Chan WCW (2008) Assessing the effect of surface chemistry on gold nanorod uptake, toxicity, and gene expression in mammalian cells. *Small* 4(1):153–9.

15. Treuel L, Jiang X, Nienhaus GU (2013) New views on cellular uptake and trafficking of manufactured nanoparticles. *J R Soc Interface* 10(82):20120939.

16. Brown JM, Giaccia AJ (1998) The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. *Cancer Res* 58(7):1408–16.

17. Junttila MR, de Sauvage FJ (2013) Influence of tumour micro-environment heterogeneity on therapeutic response. *Nature* 501(7467):346–54.

18. Rosenblum D, Peer D (2013) Omics-based nanomedicine: The future of personalized oncology. *Cancer Lett* 352(1):126–136.

19. McMillin DW, Negri JM, Mitsiades CS (2013) The role of tumour-stromal interactions in modifying drug response: challenges and opportunities. *Nat Rev Drug Discov* 12(3):217–28.

20. Thurber GM, Schmidt MM, Wittrup KD (2008) Factors determining antibody distribution in tumors. *Trends Pharmacol Sci* 29(2):57–61.

21. Stylianopoulos T, et al. (2010) Diffusion of particles in the extracellular matrix: the effect of repulsive electrostatic interactions. *Biophys J* 99(5):1342–1349.

22. Lu P, Weaver VM, Werb Z (2012) The extracellular matrix: a dynamic niche in cancer progression. *J Cell Biol* 196(4):395–406.

23. Rich L, Whittaker P (2005) Collagen and picrosirius red staining: a polarized light assessment of fibrillar hue and spatial distribution. *Braz J Morphol Sci* 22:97–104.

24. Chen X, Nadiarynkh O, Plotnikov S, Campagnola PJ (2012) Second harmonic generation microscopy for quantitative analysis of collagen fibrillar structure. *Nat Protoc* 7(4):654–69.

25. Brown E, et al. (2003) Dynamic imaging of collagen and its modulation in tumors in vivo using second-harmonic generation. *Nat Med* 9(6):796–800.

26. Sykes EA, Chen J, Zheng G, Chan WCW (2014) Investigating the impact of nanoparticle size on active and passive tumor targeting efficiency. *ACS Nano* 8(6):5696–706.

27. Perrault SD, Walkey C, Jennings T, Fischer HC, Chan WCW (2009) Mediating tumor targeting efficiency of nanoparticles through design. *Nano Lett* 9(5):1909–15.

28. Perrault SD, Chan WCW (2009) Synthesis and surface modification of highly monodispersed, spherical gold nanoparticles of 50-200 nm. *J Am Chem Soc* 131(47):17042–3.

29. Chou LYT, Chan WCW (2012) Fluorescence-tagged gold nanoparticles for rapidly characterizing the size-dependent biodistribution in tumor models. *Adv Healthc Mater* 1(6):714–21.

30. Provenzano PP, et al. (2008) Collagen density promotes mammary tumor initiation and progression. *BMC Med* 6(1):11.

31. Dudley DT, et al. (2014) A 3D matrix platform for the rapid generation of therapeutic anti-human carcinoma monoclonal antibodies. *Proc Natl Acad Sci U S A* 111(41):14882–7.

32. Stylianopoulos T, Diop-Frimpong B, Munn LL, Jain RK (2010) Diffusion anisotropy in collagen gels and tumors: the effect of fiber network orientation. *Biophys J* 99(10):3119–3128.

33. Jakobsen I, Lyng H, Kaalhus O, Rofstad EK (1995) MRI of human tumor xenografts in vivo: proton relaxation times and extracellular tumor volume. *Magn Reson Imaging* 13(5):693–700.

34. Krol A, Maresca J, Dewhirst MW, Yuan F (1999) Available Volume Fraction of Macromolecules in the Extravascular Space of a Fibrosarcoma : Implications for Drug Delivery Available Volume Fraction of Macromolecules in the Extravascular Space of a Fibrosarcoma : Implications for Drug Delivery 1. 4136–4141.

35. Walkey CD, Olsen JB, Guo H, Emili A, Chan WCW (2012) Nanoparticle size and surface chemistry determine serum protein adsorption and macrophage uptake. *J Am Chem Soc* 134(4):2139–2147.

36. Varin A, Mukhopadhyay S, Herbein G, Gordon S (2010) Alternative activation of macrophages by IL-4 impairs phagocytosis of pathogens but potentiates microbial-induced signalling and cytokine secretion. *Blood* 115(2):353–62.

37. Wang B, et al. (2011) Transition of tumor-associated macrophages from MHC class II(hi) to MHC class II(low) mediates tumor progression in mice. *BMC Immunol* 12(1):43.

38. Albanese A, Tang PS, Chan WCW (2012) The effect of nanoparticle size, shape, and surface chemistry on biological systems. *Annu Rev Biomed Eng* 14:1–16.

39. Dawidczyk CM, Russell LM, Searson PC (2014) Nanomedicines for cancer therapy: state-of-the-art and limitations to pre-clinical studies that hinder future developments. *Front Chem* 2(August):69.

40. Josefsson A, et al. (2005) Tumor size, vascular density and proliferation as prognostic markers in GS 6 and GS 7 prostate tumors in patients with long follow-up and non-curative treatment. *Eur Urol* 48(4):577–83.

41. Wisnivesky JP, Yankelevitz D, Henschke CI (2004) The effect of tumor size on curability of stage I non-small cell lung cancers. *Chest* 126(3):761–5.

42. Yu K-D, et al. (2012) Effect of large tumor size on cancer-specific mortality in node-negative breast cancer. *Mayo Clin Proc* 87(12):1171–80.

FIGURE CAPTIONS

Fig. 1. Summary of the pathophysiological changes in tumours during growth. Graphs depict the changes in vascular density (A), the proportion of tumours occupied by ECM components (B), acellular space (C), and cellular density (D) associated with tumour volume. All values were normalized to tumour cross-sectional area.

Fig. 2. Structural changes to type I collagen associated with tumour size. (**A**) Representative bright-field images of Picrosirius red stained sections that depict the evolution of collagen with tumour size. (**B**) Representative SHG microscopy images of collagen (green) overlaid with DRAQ5-stained nuclei (blue). (**C**) Graph delineating how Picrosirius red intensity fades with rising tumour volume as collagen fibrils convert to lesser organized constructs. (**D**) Histograms of SHG intensity in collagen enriched zones validates that type I collagen becomes increasingly amorphous with tumour enlargement. Scale bars represent 50 μm

Fig. 3. Results delineating how tumour uptake of AuNPs varies with tumour volume. (**A**) Bar graph of calculated AUC measurements for AuNP uptake by tumours. Yellow dotted line denotes our defined successful accumulation threshold. Overall, AuNP accumulation increases with tumour volume. (**B**) Total AuNP content in tumours of different volumes as measured by ICP-AES at 24 HPI. Results were normalized to injection dose per gram of tumour. (**C**) Bar graph summarizing how the speed of AuNP uptake varies with increasing tumour volume and particle diameter. Uptake rates remain constant for tumour volumes above 0.5 cm^3 apart for 45 nm AuNPs. Error bars denote standard error of mean values ($n > 3$). Asterisks denote statistically significant data (two-way ANOVA, $p = 0.05$).

Fig. 4. *In vitro* collagen hydrogel model of AuNP transport through tumour ECM. (**A**) Schematic depicting the *in vitro* setup used to profile AuNP infiltration into type I collagen hydrogels. (**B**) Illustration of the observed AuNP (red) infiltration process for the collagen hydrogels (green). AuNPs first concentrate at the gel-reservoir interface dependent on particle size and collagen density. Once an equilibrium is reached between AuNPs in the matrix and interface, the AuNP-front gradually diffuses deeper into the hydrogel. (**C**) Bar graph depicting the permeation of AuNPs within the collagen hydrogels at 900 minutes post-exposure. (**D**) Whisker plot depicting the cumulative results of AuNP penetration from blood vessels into tumour tissues at 24 HPI. No differences were found between tumour sizes. Bar graphs (**E**) and (**F**) summarize the differences in AuNP entry and exit from hydrogels based on collagen density and AuNP diameter. Error bars denote standard error of the mean for n = 3. Asterisks denote statistically significant data (two-way ANOVA, $p = 0.05$).

Fig. 5. Monte Carlo models simulating the dynamics of AuNP transport through and interactions with collagen matrices. (**A**) Pictorial representation of AuNP random walk in two dimensions within collagen pores. Number of collisions with the pore was tracked as measure of AuNP interactions with collagen matrices. (**B**) Representative images of simulated hydrogels of varying collagen densities in three dimensions. Images were rendered in Matlab using the same algorithms employed for assessment of AuNP diffusion through collagen matrices three dimensions. (**C**) Bar graph comparing the rate of AuNP collisions with collagen matrices of varying pore size obtained from two-dimensional simulations. Collision frequency decreases with increasing pore and AuNP size. Asterisks denotes scenario whereby AuNP size exceeded the dimensions of the pore.

(**D**) Line graph depicting the simulated changes to AuNP diffusion rate in collagen gels as collagen density increases. Collagen density did not appear to impact AuNP diffusivity but was instead dictated by AuNP size.

Fig. 6. Proposed method of selecting AuNPs according to tumour maturity. (**A**) Pseudo-colored heat maps qualitatively depict the utility of each AuNP diameter for therapeutic (left) and diagnostic (right) applications predicted by our proposed decision matrices. Rankings for particle utility for a given tumour volume have been rated from high (red) to low (green). Tabular values were calculated by taking the weighted sum of empirically ranked tumour accumulation potential, uptake rate, contrast, and permeation data according to AuNP diameter and tumour size. (**B**) Weighted importance (μ) of decision matrix parameters for application of nanoparticles to tumour diagnosis and treatment. (**C**) Flow diagram illustrating a proposed method of personalizing AuNP selection in the clinic for cancer detection and treatment.

Fig. 7. Blinded study assessing passive AuNP targeting of prostate tumours. (**A**) Whole animal fluorescent images of mice bearing orthotopic prostate tumours. Bright regions highlight areas of AuNP accumulation. (**B**) Magnetic resonance images used to confirm the presence and size of prostate tumours in mice. Dotted circles demarcate the location of the tumour. Graphs (**C**), (**D**) and (**E**) respectively compare the ICP-AES measured accumulation, tumour uptake rates, and tumour contrast of 15 and 100 nm AuNPs in small and large tumours. Error bars in all graphs denote standard error mean values for n = 3. Asterisks denote statistically significant data (two-way ANOVA, $p = 0.05$).