Abstract: Delivery of chemotherapeutic drugs to tumours by reformulation as nanoparticles has often been proposed as a means of facilitating increased selective uptake, exploiting the increased permeability of the tumour vasculature. However realisation of this improvement in drug delivery in cancer patients has met with limited success. We have compared tumour uptake of soluble Tc99m-pertechnetate and a colloid of nanoparticles with a Tc99m core, using both intravenous and intra-arterial routes of administration in a rabbit liver VX2 tumour model. The radiolabelled nanoparticles were tested both in untreated and cationised form. The results from this tumour model in an internal organ show a marked advantage in intra-arterial administration over the intravenous route, even for the soluble isotope. Tumour accumulation of nanoparticles from arterial administration was augmented by cationisation of the nanoparticle surface with histone proteins, which consistently facilitated selective accumulation within microvessels at the periphery of tumours.
List of Additions and Changes in the Revised Manuscript

1. Synthesis of nanoparticles

In the original manuscript the reader was referred to an Australian patent for information on the method of synthesis of the nanoparticles, and a very brief statement was included of the method involved.

In the revised manuscript the reader is referred to a recently granted and published US patent 8,778,300 that gives a complete and detailed description of the method of synthesis [reference 11]. This reference is readily available on the public internet database of the USPTO. In addition, it is explained that the technology is based on the Technegas™ lung diagnostic aerosol, which is also referenced with the granted and published US patent 7,722,856 [new reference 12]. The included brief description of the method of synthesis has also now been expanded to include the salt sublimation and isotope ablation conditions.

2. Characterisation of nanoparticles

The nature of the nanoparticles has previously been well characterised by Senden et al; new reference [13]. These studies, including electron microscopy, established that the nanoparticles consist of metallic platelets encapsulated by multiple lamellae of carbon. This information and also later vapour phase particle sizing data has been added to the Methods section to give the reader much more material information about the physicochemical features of the nanoparticles.

3. Surface properties of nanoparticles

We have previously published on the ability of these nanoparticles to strongly bind polycations on their surface, thus producing marked changes in their adhesion properties in a membrane binding model and in microwell binding assays [reference 15]. The mechanism of binding is proposed to be a multi-site pi-cation interaction between the positively charged amino groups of polycations and the pi electrons of the planar carbon rings in graphite. This information is now detailed in a separate new paragraph of the Methods section titled "Coating of FL with polycations", including new references [16 and 17] to the relevant original publications by other researchers on the pi-cation interaction. We also give further information relating to histone proteins [new reference 18] that represent a special class of highly polycationic proteins, and the studies we have previously published showing that histones bind strongly to the nanoparticles, reversible only with a strong ionic detergent [19]. These previous publications [15 and 19] also provide detailed studies of the biological properties of FL and its coated forms, notably the mechanism of accumulation of polycation coated FL in the capillary network of the lungs, and its inhibition by competing polycations.

4. Preparation of polycation coated nanoparticles

The details of binding conditions have been added in the new paragraph on coating of FL with polycations.
Listing of Six additional new References included in the Revised Manuscript


**Full Title:** The uptake of soluble and nanoparticulate imaging isotope in model liver tumours after intra-venous and intra-arterial administration

**Authors’ names and academic degrees:** Ross W Stephens¹ (PhD), Karen J Knox¹, Lee A Philip², Kelly M Debono², Jessica L Bell¹ (BSc), David W King¹, Christopher R Parish³ (PhD), Tim J Senden¹ (PhD), Marcel R Tanudji⁴ (PhD), Jillean G Winter⁴ (BTh), Stephanie A Bickley⁴ (BSc), Michael J Tapner⁴ (B App Sc), Jian H Pang⁴ (MD, PhD) and Stephen K Jones⁴ (PhD)

**Affiliations:** ¹Biomedical Radiochemistry Laboratory, Department of Applied Mathematics, Research School of Physics and Engineering, Australian National University, ²Animal Services Division, Research School of Biology, Australian National University; ³Vascular Biology Laboratory, John Curtin School of Medical Research, Australian National University, ⁴Sirtex Medical Ltd, Sydney, Australia.

**Short title:** Uptake of imaging isotope in liver tumours

**Corresponding author:** Professor Ross W. Stephens

**Address:** Department of Applied Mathematics, Research School of Physical Sciences and Engineering, Australian National University, Canberra ACT 0200, Australia.

**Email:** ross.stephens@anu.edu.au

**Conflicts of interest statement:** The ANU authors are financially supported by Sirtex Medical Ltd (Sydney).

**Sources of support for this research:** Sirtex Medical Ltd, Sydney Australia.

**Number of figures:** 4
Abstract:

Delivery of chemotherapeutic drugs to tumours by reformulation as nanoparticles has often been proposed as a means of facilitating increased selective uptake, exploiting the increased permeability of the tumour vasculature. However, realisation of this improvement in drug delivery in cancer patients has met with limited success. We have compared tumour uptake of soluble Tc99m-pertechnetate and a colloid of nanoparticles with a Tc99m core, using both intravenous and intra-arterial routes of administration in a rabbit liver VX2 tumour model. The radiolabelled nanoparticles were tested both in untreated and cationised form. The results from this tumour model in an internal organ show a marked advantage in intra-arterial administration over the intravenous route, even for the soluble isotope. Tumour accumulation of nanoparticles from arterial administration was augmented by cationisation of the nanoparticle surface with histone proteins, which consistently facilitated selective accumulation within microvessels at the periphery of tumours.

Keywords: Nanoparticle delivery, arterial administration, vascular permeability, imaging isotope, liver cancer.
Introduction

The growth of a tumour in a host organ is accompanied by angiogenesis that not only markedly changes the architecture of the vascular network [1-3], but also considerably increases access to the extravascular space due to the increased permeability of the new vessels [4]. This provides opportunity for improvement in delivery of chemotherapeutic agents by reformulation into nanoparticulate composites that can extravasate into tumours [5].

Nanoparticles tend to be rapidly removed from the circulation by the phagocytic cells resident in the liver and spleen (reticuloendothelial system; RES), but it has been suggested they can also escape through the highly fenestrated walls of tumour capillaries and distribute within the tumour matrix [6]. Extravasation of macromolecules into tumours has been known for many years, visualised earlier for example by increased dye uptake [7], and this has become known as the enhanced permeability and retention (EPR) effect [8]. Thus it is the object of much current research to exploit tumour EPR by administration of nanoparticulate formulations of cytotoxic agents [9, 10]. It should be noted however that while most preclinical studies have been done in animal models of subcutaneously implanted tumours, data on extravasation, penetration and accumulation of nanoparticles in tumours of internal organs, like many of those cancers occurring in human patients, is especially lacking. In this context, it is also important to explore different routes of administration, especially intra-arterial instillation.

In this report we have investigated tumour uptake of imaging radioisotope in the form of a soluble compound (Tc99m-pertechnetate) and compared this with uptake of a nanoparticulate isotope preparation. Further, we have performed these imaging investigations in a rabbit tumour model of liver cancer, where we could study biodistribution inside this internal organ after intra-venous and intra-arterial administration of both types of agents.
Materials and Methods:

Nanoparticle synthesis

Carbon-caged Tc-99m, (FibrinLite; FL) was synthesised as described in detail in US patent 8,778,300 [11]. The nanoparticle technology employed was based on Technegas™, a radioactive aerosol preparation developed for diagnostic ventilation imaging of the lungs [12]. Vapour-phase particle sizing using an electrostatic particle classifier (TSI Inc, MN USA) showed the aerosol comprises log-normal distributed particles with the bell curve centred on 150-350 nm, and negligible particles below 100 nm or above 400 nm. Electron microscope characterisation shows metallic platelets surrounded by multiple lamellae of carbon [13]. Using the patented FL process, sodium Tc99m-pertechnetate solution was loaded into a graphite crucible and after removing sodium chloride by sublimation at 1650°C, the isotope was plasma ablated at 2750°C into an argon gas stream. Aerosol nanoparticles were collected into water (6.0 mL) from the gas stream using a Browitt sonicating precipitator [14], thus producing a stable colloidal dispersion of FL. The radioactive FL colloid was filtered through a 450 nm hydrophilic membrane (mixed cellulose ester (MCE); Millipore) before use. A typical preparation of FL contained approximately 5 µg/mL of graphitic carbon with a specific activity of 20 MBq/µg. FL nanoparticles are highly stable, and integrity of the isotope encapsulation is preserved under standard autoclave conditions of 20 min at 120°C.

Coating of FL with polycations

We have previously shown using a membrane filtration model and microwell binding assays that polycations such as polylysine bind to the surface of FL with high affinity [15]. We have proposed [15] that this is mediated by multi-site pi-cation interactions [16,17] between the positively charged amino groups of the amino acid side-chains and the pi-electrons of the planar carbon rings of the graphite surface. The histone family of proteins are also polycations due to a high content of arginine and lysine residues [18] and binding of these proteins to FL can only be reversed with strong ionic detergents such as sodium dodecyl sulfate [19]. Binding of these polycations to FL is also stable under in vivo conditions, as shown by our
previous imaging studies of polycation coated FL in the capillary network of rabbit lungs [15, 19].

In the present study, FL was treated with a selection of polycations, including protamine sulphate (PS; 20 µg/mL), poly-D-lysine (PDL; 6 µg/mL) and calf thymus histone proteins (CTH; 10 µg/mL), all from Sigma Aldrich, Castle Hill, Sydney. FL was buffered with 0.5 mM Tris acetate buffer (pH 7.2) before addition of polycations, which were then allowed to bind for 1 h at 20ºC before use of the coated FL preparations in animal imaging experiments.

**Rabbit VX2 liver tumour model**

All rabbit procedures adhered to the National Health and Medical Research Council's Animal Welfare Code for the appropriate use of animals for scientific purposes (Australian Government, 7th Ed. 2004), and the experimental protocols were approved by the Australian National University (ANU) Animal Ethics Committee. New Zealand white rabbits were used at a minimum age of four months, when their body weight was an average of 3.8 +/- 0.6 kg (n = 23) and their livers weighed 117 +/- 24 grams. The transplantable rabbit VX2 tumour [20] was a kind gift of Dr J Geschwind (Johns Hopkins University, Baltimore, USA) and was maintained as a serial transplant on the hind limbs. Liver implants of tumour tissue were made at a single site in one lobe by keyhole surgery under ventilation anaesthesia with isoflurane, and allowed to grow for 18 days before use of the rabbit in imaging experiments. At this stage of growth the tumour had a diameter of less than 2 cm, and the oblate ellipsoid of tumour was still completely contained within the liver lobe and did not involve the body wall or other organs. Macroscopically, the tumour usually had a necrotic centre, with an enhanced blood supply evident in an actively growing vascularised peripheral zone. Measurements of the shortest and longest axes of the excised tumours were used to estimate the volume, which averaged 2.9 +/- 1.9 cm³ (n = 41).

**Biodistribution studies of radiolabelled materials in rabbits**

Rabbit imaging studies were done under ventilation anaesthesia with isoflurane using either a Siemens Diacam gamma camera or a GE Hawkeye Infinia SPECT-CT camera. For imaging studies with intravenous administration, sodium Tc99m-pertechnetate or FL colloid (3
mL, 130-170 MBq) was injected in an ear vein. Intrahepatic artery instillations of pertechnetate and FL were performed by catheterisation of the cystic artery and using pulses of the isotope material (total 5 mL, 130-170 MBq) interspaced with normal hepatic artery blood flow, so as to disperse the isotope throughout the liver with close to normal blood perfusion conditions [21]. Symporter uptake of pertechnetate in organs and tumours was tested for by prior intravenous injection of sodium perchlorate (3 mg/kg; Sigma Aldrich) [22]. Heparan sulphate mediated endothelial uptake of cationised FL was tested for by prior intravenous injection of competing polyions [19]: PS (1 mg/kg), PDL (1 mg/kg), and heparin (1 mg/kg; Sigma Aldrich), or by prior treatment of the rabbit for 1 h with intravenous injection of heparinase type III (10 units; Sigma Aldrich) [19].

Static 5 min acquisitions were made on a 1024 x 1024 matrix approx 10 min after administration of the imaging agent and again after one hour. After completion of imaging, the rabbit was euthanized by lethal injection while still under anaesthesia, and the liver was excised after tying off all vessels to prevent leakage of isotope. The excised liver was then imaged separately using a 5 min acquisition on a 1024 x 1024 matrix, and utilising a zoom function (e.g. 3.2x or 4x). Counts registered in the acquisitions were corrected for the background activity of the corresponding field, and the corrected counts were used for calculation of the percentage activity in the liver compared to the carcass. Tumour uptake data was limited to tumours that had grown to at least 1.0 $\text{cm}^3$ and located in the same lobe of the liver in each rabbit. Activity levels in the images shown in the Figures were assigned false colours using the A2 colours in the Siemens Diacam gamma camera or the XT21 Brain1 colours in the Hawkeye Infinia SPECT/CT colour maps respectively.
Results

Biodistribution of soluble Tc99m-Pertechnetate

Intravenous injection of soluble Tc99m-pertechnetate produced images of circulating radioactivity distributed according to the blood perfusion of the main organs (heart, lungs, liver and spleen) but also with some accumulation in the kidneys and thyroid at 1 h post-injection (Fig 1A). The excised liver 1 h post-injection retained only 15.8% of the total radioactivity (see Table 1) and the gamma camera image showed it was generally dispersed throughout the lobes of the organ. When Tc99m-pertechnetate was instilled in the hepatic artery, again only 14.5% of the activity was found in the liver after 1 h (Table 1), and the systemic distribution of label was not distinguishable from that obtained with intravenous injection of pertechnetate.

Intravenous injection of Tc99m-pertechnetate into rabbits bearing VX2 implants in their livers produced images with a noticeable shadow (isotope deficit) at the site of the tumour implant compared to the rest of the liver (Fig 1B). Retention of Tc99m-pertechnetate label by the liver hosting a tumour was similar to a normal liver; 12.3% of the total radioactivity at 1 h post injection (Table 1).

By contrast, hepatic artery instillation of Tc99m-pertechnetate into rabbits hosting a VX2 liver tumour showed in all cases (n = 7) a prominent focal area of radioactivity in the liver persisting through to at least 1 h post instillation (Fig 1C), so that on excising the liver and imaging separately the percentage of total activity retained in the liver was clearly higher than in normal rabbits (26.4%; see Table 1). Furthermore there was a considerable accumulation of radiolabel in the tumour (Fig 1D); 28.9% of the isotope retained by the whole liver (Table 1), as estimated by applying a region of interest to the image, which usually results in an underestimate. Since pertechnetate can potentially be internalised in cells by the expression of a membrane transport system known as the sodium iodide symporter (NIS), we tested the effect of prior intravenous administration of sodium perchlorate as a specific competitive inhibitor [22]. Preadministration of perchlorate strongly attenuated thyroid uptake of Tc-99m-pertechnetate but only partially affected liver tumour uptake (Table 1) and did not prevent clear definition of liver tumours in imaging (see Fig 1E). Since polycations have been shown to increase vascular permeability in the lungs [23], we also tested the effect of intra-hepatic
artery instillation of PDL (150 µg) 15 min before instilling the Tc99m-pertechnetate in the
same catheter; this pretreatment did not increase the retention of label in the liver or the
tumour (not shown).

Biodistribution of colloidal FL

Intravenous injection of untreated FL showed rapid uptake and concentration in the
reticuloendothelial system (liver, spleen and bone marrow; RES) as reported previously [15].
Imaging of the excised liver at 1 h post injection showed that the accumulated radioactivity
was generally dispersed throughout the lobes of the organ, and accounted for approximately
80% of the total radioactivity administered (Table 2). This figure was higher than our previous
estimate of 60% made using a region of interest on the liver image in the intact animal [15].
When FL was administered by hepatic artery instillation, the whole body image and excised
liver image were closely similar to those obtained after intravenous injection of FL, but the
isotope activity retained in the liver at excision 1 h post instillation was higher, over 90% of the
total dose administered (Table 2).

Intravenous injection of untreated FL into rabbits bearing VX2 implants in their livers
showed rapid uptake of label by the liver as in normal rabbits, but on imaging the excised liver
at 1 h post injection it was evident that there was a noticeable shadow (isotope deficit) at the
site of the tumour implant compared to the rest of the liver (Figs 2A,B). When the FL was
instilled via the hepatic artery, liver uptake was as strong as seen in a normal liver (93.7%;
Table 2). In 2 out of 5 of these cases there was a noticeable arc of activity proximal to the
main arterial entry or even a complete ring of activity around the site of the tumour, but these
features did not have a remarkably higher count per pixel than some other normal areas of
the same liver (Figs 2C,D).

Biodistribution of cationised colloidal FL

It has been suggested that nanoparticles with a positively charged surface
are more readily taken up by endothelium in the vasculature of a tumour [24,25]. Thus we
tested the effect of cationising FL on its uptake into livers and liver tumours following hepatic
artery instillation. FL was cationised with surface coatings of PS, PDL and CTH [15,19].
These surface coatings did not change the strong liver retention of nanoparticles after arterial instillation (Table 2). Increased tumour uptake of nanoparticles was seen in a minority of cases after coating with PS and PDL (3 out of 10 cases), but this could have been merely due to larger tumour size; a detailed study was not done. However coating with CTH consistently produced a pronounced increase in tumour uptake of isotope, so that the tumour was often readily visible on imaging of the intact animal (Fig 3A) and in every case (n=8) featured prominently in the excised liver (Fig 3B). Radiolabel was consistently concentrated at the periphery of tumours, sometimes appearing as a continuous bright ring encircling the actual tumour mass (Fig 3B). Of the different liver tumour imaging methods tested in this study, intra-arterial administration of CTH coated FL produced the highest proportion of the administered dose accumulated at tumour sites (17.5%; Table 3), while 95% of the total administered dose was still retained by the host liver.

Since marked accumulation of CTH/FL at tumour sites was evident in gamma camera imaging, we investigated further at the histological level. In standard haematoxylin and eosin stained sections, it was not possible to find any carbon particles after hepatic artery installation of uncoated FL, even at high power magnification; the uniform distribution of the microgram quantities of nanoparticles into the rabbit livers (mean weight 117 g; n=23) produced dilution to the point where they were not detectable. By contrast, CTH/FL that was strongly accumulated in liver tumours as seen by gamma camera imaging (Fig 3B), could also be found on microscopic examination of sections from the same liver (Fig 3C). Clusters of black carbon particles were clearly visible accumulated in microvessels located in close proximity to the edge of the tumour, but were absent in vessels further away from the tumour, or within the nests of tumour cells (Fig 3C). In some cases extravascular macrophages contained some carbon particles, but it was clear that the majority of nanoparticles accumulated in intravascular clusters without extravasation.

The mechanism responsible for the liver tumour accumulation of CTH/FL was investigated using similar inhibition methods to those we previously used in lung perfusion studies [15,19]. Unlike accumulation of CTH/FL on heparan sulphate in the capillary network of normal rabbit lungs, the uptake of CTH/FL in rabbit VX2 liver tumours was not abolished by prior intravenous injection of potentially competing polycations; tumours were still clearly
visible in livers from rabbits that had been preinjected with PS (Fig 4A) and PDL (Fig 4B), and the tumours were also still labelled following pretreatment of the rabbit with intravenous heparinase [19] (not shown). Labelling of tumour was also still clearly evident even when the CTH/FL preparation was treated with heparin in vitro prior to intra-arterial instillation (Fig 4C). The percentage of CTH/FL label present at tumour sites in all of these inhibition tests was not outside the range for the tumours in Table 3.
Discussion

A detailed study of the liver uptake of soluble Tc-99m pertechnetate and Tc99m radiolabelled nanoparticles (FL) was made in rabbits following administration by both intravenous and intra-arterial (common hepatic artery) routes. Uptake from this set of administrations in normal rabbits was then compared with the uptake obtained after administration to rabbits bearing a model liver tumour, the transplantable rabbit VX2 carcinoma. This provided data for each form of the radiolabel on the accumulation within the liver compared to the systemic distribution, and on the uptake of label by a tumour compared to the normal liver tissue of the host.

While soluble Tc-99m as pertechnetate was widely distributed in the body following intravenous injection, with some accumulation in the thyroid and kidneys, FL was rapidly taken up by the reticuloendothelial system, as shown previously [15]. The presence of a VX2 tumour in the liver showed as a negative area of uptake after intravenous injection of either pertechnetate or FL. The necrotic centre of the tumour may have produced an area of less perfusion for soluble isotope, while in the case of FL the lack of Kupffer cell function in the tumour compared to the liver may have reduced active accumulation of the nanoparticles.

In rabbits hosting a liver VX2 implant, there were notable differences when pertechnetate and FL were administered arterially; pertechnetate showed unexpectedly high and persistent signal in the tumour, presumably due to the highly fenestrated new arterial vessels produced during tumour angiogenesis under the influence of tumour cytokines [26]. Active uptake of pertechnetate has been noted before in other tumours [27, 28], where it has been shown to be due to endogenous iodide symporter (NIS) expression, but in the case of VX2 liver tumours we found that while a competitive inhibitor of the NIS (perchlorate) prevented uptake of pertechnetate in the thyroid, it did not prevent pertechnetate uptake by the VX2 tumour in the liver. Permeability of the liver arterial system to pertechnetate was unaffected by PDL, unlike the increase in permeability induced in the lungs by polycations, shown by Dull et al [23] to be mediated by endothelial heparan sulphate.

Arterial administration of FL showed some concentration of label around the tumour site in a minority of cases, presumably also due to the increased vascularity following angiogenesis, but this was not as consistent or pronounced as in the case of pertechnetate,
suggesting the nanoparticles may be too large to extravasate [29]. However when the FL was surface cationised by CTH, there was a consistent and marked increase in accumulation at the tumour site. In at least two cases this accumulation was visible both in gamma camera imaging as well as in histology, when accumulations of black carbon were found inside microvessels close to the edge of tumour cell nests, but not in vessels further away.

Since accumulation of CTH/FL at liver tumours was not affected by prior treatment of the host rabbits with PDL or heparinase, or even by treatment of the CTH/FL with heparin in vitro prior to intra-arterial instillation, it appears that the mechanism involved is not a simple charge interaction with endothelial heparan sulphate as we found previously in the lungs [15,19]. Significantly, there was also no effect on liver tumour accumulation of CTH/FL when the host was pretreated with PS. These observations together suggest that a more specific interaction of CTH is involved, since the protamines are a very similar group of cationic nuclear proteins to histones. One possibility would be that CTH is binding to Toll-like receptors on the angiogenic endothelium induced by cytokines at tumour sites [30]. The results also showed that accumulation of nanoparticles at a tumour site does not necessarily imply extravasation has occurred; at histology we found accumulation of CTH/FL within microvessels at the edge of liver tumours but not outside microvessels or within the nests of tumour cells. Clearly this has significant implications not only for delivery of cytotoxic drugs to tumour cells but also for tumour cell targeting dependent on monoclonal antibody coated nanoparticles actually reaching their cognate target antigens on tumour cells. The data instead supports the notion that targeting of angiogenic endothelium at tumour sites is a more attractive strategy.
Conclusion

Our results with a model tumour system hosted in an internal organ provide useful insight into the tissue penetration of different isotope preparations following administration by the intravenous and intra-arterial routes, further illustrating the difference in tumour accessibility. Uptake of soluble pertechnetate and FL nanoparticles into tumours from the venous circulation was uniformly absent, whereas both preparations were accumulated in the immediate periphery of liver tumours following intra-arterial instillation, even despite their large difference in size. Effective dosing of liver tumours with a nanoparticle agent is thus shown to have considerably more chance of success when administered by the arterial route*. Our isotope imaging and histological findings with histone-coated nanoparticles further suggest that this formulation can specifically accumulate nanoparticles in angiogenic microvessels at the growth zone peripheral to tumours.

*Footnote: During preparation of this manuscript some similar findings were reported by Tian et al [31].
Acknowledgements:

The ANU authors acknowledge the collaborative project support generously provided by Sirtex Medical Ltd (Sydney), including donation of a GE Hawkeye Infinia SPECT/CT scanner and a Xeleris image processing system. We thank Dr Mark Williamson of Gribbles Veterinary Pathology Australia (Clayton, Victoria) for excellent histology. We also thank Gary Somerville of Cyclomedica Pty Ltd for assistance with Technegas™ equipment, and Dr Bill Burch, the inventor of Technegas™ for helpful discussions. Finally we would like to thank National Capital Diagnostic Imaging (Canberra) for donation of a Siemens Diacam gamma camera.
References:


Figure captions

Figure 1. Tc99m-pertechnetate biodistribution in normal rabbits and in rabbits with VX2 tumour implants. The gamma camera images show radioisotope activity in the liver of a normal rabbit (Fig 1A) and livers of rabbits hosting a VX2 implant (Figs 1B,C,D,E), 1 h after administration of Tc99m-pertechnetate (130 MBq) by intravenous injection (3 mL; Figs 1A,B) or intra-arterial instillation (5 mL; Figs 1C,D,E). Organs in Fig 1A are labelled as thyroid (T), heart (H), liver (L) kidneys (K) and bladder (B). Note the negative image of the VX2 tumour (indicated by V) in the liver in Fig 1B, and compare with the positive imaging of the tumour (V) in the intact rabbit in Fig 1C, and in the excised liver from Fig 1C shown in Fig 1D. Positive imaging of the tumour in a rabbit by intra-arterial Tc99m-pertechnetate was not abolished (V in Fig 1E) by prior intravenous injection of sodium perchlorate (3 mg/kg), an inhibitor of the active transport of iodide and pertechnetate [22]. Images were acquired with a GE Hawkeye Infinia gamma camera.

Figure 2. Uptake of FL in rabbit livers hosting VX2 tumours. The gamma camera images show radioisotope activity in the excised livers of rabbits 1 h after intravenous injection (3 mL; Figs 2A and 2B) and intra-arterial (5 mL; Figs 2C and 2D) instillation of untreated FL (130 MBq). Note the negative image of the tumours indicated by V in Figs 2A and 2B, and contrast with the positive signal obtained at the tumours in Figs 2C and 2D. Images were acquired with a Siemens Diacam gamma camera.

Figure 3. Uptake of histone-coated FL in rabbit liver hosting VX2 tumour. The gamma camera images show radioisotope biodistribution in an intact rabbit (Fig 3A) and in the corresponding excised liver from this rabbit (Fig 3B), 1 h after intra-arterial instillation of CTH/FL (5 mL, 130 MBq). Note the strong labelling of the liver region hosting the tumour visible in the intact animal and in the excised liver (tumour indicated by V). Images 3A and 3B were acquired with a Siemens Diacam gamma camera. Figure 3C shows a haematoxylin and eosin stained section of the liver VX2 tumour from the same rabbit liver. Note accumulations of black carbon nanoparticles in the microvessels at the edge of a nest of tumour cells. Magnification x200.
Figure 4. Inhibition tests of tumour CTH/FL uptake. The excised liver images shown are from rabbits injected intravenously with protamine sulphate (1 mg/kg) (Fig 4A) and poly-D-lysine (1 mg/kg) (Fig 4B), 10 min prior to intra-arterial instillation of CTH/ FL (5 mL, 130 MBq). Note that tumours were labelled in each of these pretreated rabbits (tumours indicated by V). When the CTH/FL preparation was treated with heparin (1 mg/mL) in vitro before intra-arterial instillation, the tumour was still strongly labelled (Fig 4C). Images in Figs 4A-C were acquired with a GE Hawkeye Infinia gamma camera.
Table 1: Tc99m-Pertechnetate uptake in normal rabbit livers and livers with VX2 implants

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Admin Route</th>
<th>Mean Activity in Liver (%Total)</th>
<th>Number of Tests</th>
<th>Range (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>IV</td>
<td>12.9</td>
<td>2</td>
<td>10.0 - 15.8</td>
</tr>
<tr>
<td></td>
<td>IHA</td>
<td>14.5</td>
<td>2</td>
<td>14.5 – 14.5</td>
</tr>
<tr>
<td>VX2</td>
<td>IV</td>
<td>12.3</td>
<td>2</td>
<td>12.1 – 12.5</td>
</tr>
<tr>
<td></td>
<td>IHA</td>
<td>26.4</td>
<td>5</td>
<td>17.6 – 34.6</td>
</tr>
<tr>
<td></td>
<td>IV perchlorate then IHA pertechnetate</td>
<td>19.2</td>
<td>2</td>
<td>16.4 – 22.0</td>
</tr>
</tbody>
</table>

Retention of radioisotope activity in the livers of normal rabbits and livers of rabbits hosting a VX2 implant, 1 h after administration of Tc99m-pertechnetate (130 MBq) by intravenous injection (3 mL; IV) or intra-arterial instillation (5 mL; IHA). Activity in the excised liver image was compared to the body image after removal of the liver, and corrected for background in the corresponding detection fields. For two tumour rabbits, sodium perchlorate (3 mg/kg) was injected intravenously before IHA instillation of the isotope, to test the role of symporter in tumour retention of pertechnetate [22].
Table 2: FL uptake in normal livers and livers with VX2 implants

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Admin Route</th>
<th>Mean Activity in Liver (%Total)</th>
<th>Number of Tests</th>
<th>Range (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>IV</td>
<td>79.1</td>
<td>3</td>
<td>75.8 – 81.2</td>
</tr>
<tr>
<td></td>
<td>IHA</td>
<td>91.5</td>
<td>5</td>
<td>89.4 – 94.4</td>
</tr>
<tr>
<td>VX2</td>
<td>IV</td>
<td>74.3</td>
<td>2</td>
<td>73.0 – 75.7</td>
</tr>
<tr>
<td></td>
<td>IHA</td>
<td>93.7</td>
<td>5</td>
<td>91.5 – 95.3</td>
</tr>
<tr>
<td></td>
<td>IHA PS/FL</td>
<td>92.9</td>
<td>2</td>
<td>90.7 – 95.1</td>
</tr>
<tr>
<td></td>
<td>IHA PDL/FL</td>
<td>96.2</td>
<td>8</td>
<td>95.0 – 97.1</td>
</tr>
<tr>
<td></td>
<td>IHA CTH/FL</td>
<td>95.0</td>
<td>9</td>
<td>87.4 – 98.0</td>
</tr>
</tbody>
</table>

Retention of radioisotope activity in the liver of normal rabbits and livers of rabbits hosting a VX2 implant, 1 h after administration of FL nanoparticles (130 MBq) by intravenous injection (3 mL; IV) or intra-arterial instillation (5 mL; IHA). Activity in the excised liver image was compared to the body image after removal of the liver, and corrected for background in the corresponding detection fields. FL was tested either untreated or surface cationised with protamine sulphate (PS), poly-D-lysine (PDL) or calf thymus histones (CTH).
Table 3: Liver and VX2 tumour uptake of intra-arterial pertechnetate and CTH/FL

<table>
<thead>
<tr>
<th>Measure</th>
<th>Tc99m-Pertechnetate n = 7</th>
<th>CTH/FL n = 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>% ID in Liver</td>
<td>24.4 +/- 7.7</td>
<td>94.7 +/- 3.5</td>
</tr>
<tr>
<td>% Liver activity in tumour</td>
<td>28.9 +/- 6.8</td>
<td>18.6 +/- 5.0</td>
</tr>
<tr>
<td>% ID in tumour</td>
<td>7.2 +/- 3.4</td>
<td>17.5 +/- 5.0</td>
</tr>
<tr>
<td>Estimated % ID/g liver*</td>
<td>0.15</td>
<td>0.66</td>
</tr>
<tr>
<td>Estimated % ID/g tumour**</td>
<td>2.5</td>
<td>6.0</td>
</tr>
<tr>
<td>Estimated ratio tumour/liver</td>
<td>17</td>
<td>9.1</td>
</tr>
</tbody>
</table>

*Mean liver weight = 117 +/- 24 g (n = 23)

** Mean tumour weight = 2.9 +/- 1.9 g (n = 41)

Retention of radioisotope activity in the livers of rabbits hosting a VX2 implant, 1 h after administration of Tc99m-pertechnetate or CTH/FL nanoparticles (5 mL, 130 MBq) by intra-arterial instillation (IHA). The percentage of the administered dose (ID) in the liver was obtained from the image of the excised liver and the image of the body after removal of the liver, and the percentage of ID retained in the tumour was estimated by drawing a region of interest over the tumour in the image of the excised liver. Mean values corrected for background in the corresponding detection fields are shown with the standard deviation. The estimate shown for % ID/g liver is based on a mean weight of 117 g obtained for 23 excised livers, while the estimated % ID/g tumour is calculated from measurements of the largest and smallest diameters of 41 sectioned tumours from excised livers.