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(54)【発明の名称】温度依存性造影剤による強化型超音波検出

(57)【要約】
【課題】増強されたイメージング（画像化）、又は薬剤送達、温熱療法や寒冷療法などの治療方法若しくはその他の画像化様式と共に用いられる超音波画像化方法及び装置。
【解決手段】超音波標的（ターゲット）に結合する温度依存性造影剤の温度及び超音波反射率の変化を引き起こすことにより超音波検出を増強する。

【特許請求の範囲】
【請求項１】
（１）超音波ターゲットに結合しかつ温度の変化に応じて音響反射率の変化を引き起こす非気体性音響画像化物質を該超音波ターゲットに投与すること、及び
（２）前記超音波ターゲットと結合した前記非気体性音響画像化物質の音響反射率に測定可能な変化を引き起こすよう温度を変化させること
を含むことを特徴とする超音波ターゲットの音響反射率の変化方法。
【請求項２】
前記非気体性音響画像化物質は、液体フルオロカーボンを含むエマルジョンから構成されることを特徴とする請求項１に記載の方法。
【請求項３】
前記フルオロカーボンは、ペルフルオロオクタンであることを特徴とする請求項２に記載の方法。
【請求項４】
前記非気体性音響画像化物質は、前記超音波ターゲットに結合するリガンドを有することを特徴とする請求項１に記載の方法。
【請求項５】
前記リガンドは、抗体、抗体のフラグメント、ポリペプチド、ペプチド様物質、多糖、アプタマー、脂質、核酸、又はレクチンであることを特徴とする請求項４に記載の方法。
【請求項６】
前記非気体性音響画像化物質は、ビオチン剤と結合したリガンド、ビオチン剤と結合したエマルジョン、及び該ビオチン結合リガンド及び該ビオチン結合エマルジョンに固定化されたアビジン剤を含むことを特徴とする請求項５に記載の方法。
【請求項７】
前記非気体性音響画像化物質は、エマルジョンに固定化されたプライマー物質に固定化されたリガンドを含むことを特徴とする請求項５に記載の方法。
【請求項８】
前記エマルジョンは、更に、生理活性剤を含むことを特徴とする請求項１に記載の方法。
【請求項９】
前記生理活性剤は、抗悪性腫瘍剤、放射性医薬製剤、ホルモン、鎮痛剤、非ステロイド系抗炎症剤、麻酔剤、鎮静剤、神経筋遮断剤、抗菌剤、抗寄生虫剤、抗ウイルス剤、インターフェロン、硝酸塩、 ざ瘡製剤、アンドロゲン剤、抗糖尿病剤、抗痛風剤、抗ヒスタミン剤、鎮咳剤、鬱血除去剤、去痰剤、抗潰瘍剤、下剤、抗凝血剤、免疫化剤、抗痙攣剤、抗パーキンソン剤、エストロゲン様製剤、甲状腺製剤、又は鉄含有抗貧血剤であること
を特徴とする請求項８に記載の方法。
【請求項１０】
前記非気体性音響画像化物質は、更に、磁気共鳴画像化物質、電子スピン共鳴画像化物質、分光画像化物質、陽電子放射断層撮影用画像化物質、光学画像化物質、Ｘ線画像化物質、核医学用画像化物質、又はこれらの組み合わせを含むことを特徴とする請求項１に記載の方法。
【請求項１１】
前記分光画像化物質は、核磁気共鳴分光画像化物質又はラマン分光画像化物質を含むことを特徴とする請求項１０に記載の方法。
【請求項１２】
前記非気体性音響画像化物質は、常磁性又は超常磁性素子、放射性核種、又は光活性剤を含むことを特徴とする請求項１１に記載の方法。
【請求項１３】
前記温度の変化は、前記結合した非気体性音響画像化物質を活性化して、該結合非気体性音響画像化物質の温度を上昇し、かつその表面の音響反射率を増加することを含むことを特徴とする請求項１に記載の方法。

【請求項１４】
前記活性化は、超音波、短波、マイクロ波、磁気照射、電磁エネルギー、又はこれらの組み合わせからエネルギーを生成することによって行なわれることを特徴とする請求項１３に記載の方法。
【請求項１５】
前記結合非気体性音響画像化物質の温度の変化は、該結合非気体性音響画像化物質の温度を低下して、前記超音波ターゲットの音響反射率の測定可能な低下を引き起こすことを含むことを特徴とする請求項１に記載の方法。
【請求項１６】
前記結合非気体性音響画像化物質の温度の低下は、寒冷療法又は心臓バイパス形成手術の一環として実行されることを特徴とする請求項１５に記載の方法。
【請求項１７】
前記温度の変化は、少なくとも５℃毎の前記結合非気体性音響画像化物質の温度の変化を含むことを特徴とする請求項１に記載の方法。
【請求項１８】
（１）超音波ターゲットに結合しかつ温度の変化に応じて音響反射率の変化を引き起こす非気体性音響画像化物質を該超音波ターゲットに投与すること、
（２）前記超音波ターゲットと結合した前記非気体性音響画像化物質の音響反射率に測定可能な変化を引き起こすよう温度を変化させること、及び
（３）前記結合した非気体性音響画像化物質の音響反射率の変化を検出すること
を含むことを特徴とする超音波ターゲットの増強された音響反射率の測定方法。
【請求項１９】
前記検出は、
（ａ）前記結合非気体性音響画像化物質の温度を変化する前に反射率を測定すること、
（ｂ）前記結合非気体性音響画像化物質の温度を変化した後に反射率を測定すること、及び
（ｃ）前記結合非気体性音響画像化物質の温度を変化する前の反射率に対する前記結合非気体性音響画像化物質の温度を変化した後の反射率の変化を決定すること
を含むことを特徴とする請求項１８に記載の方法。
【請求項２０】
前記非気体性音響画像化物質は、液体フルオロカーボンを含むエマルジョンから構成されることを特徴とする請求項１９に記載の方法。
【請求項２１】
前記フルオロカーボンは、ペルフルオロオクタンであることを特徴とする請求項２０に記載の方法。
【請求項２２】
前記非気体性音響画像化物質は、前記超音波ターゲットに結合するリガンドを有することを特徴とする請求項１９に記載の方法。
【請求項２３】
前記リガンドは、抗体、抗体のフラグメント、ポリペプチド、ペプチド様物質、多糖、アプタマー、脂質、核酸、又はレクチンであることを特徴とする請求項２２に記載の方法。
【請求項２４】
前記非気体性音響画像化物質は、ビオチン剤と結合したリガンド、ビオチン剤と結合したエマルジョン、及び該ビオチン結合リガンド及び該ビオチン結合エマルジョンに固定化されたアビジン剤を含むことを特徴とする請求項２３に記載の方法。
【請求項２５】
前記非気体性音響画像化物質は、エマルジョンに固定化されたプライマー物質に固定化されたリガンドを含むことを特徴とする請求項２３に記載の方法。
【請求項２６】
前記エマルジョンは、更に、生理活性剤を含むことを特徴とする請求項１９に記載の方法

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【請求項２７】
前記生理活性剤は、抗悪性腫瘍剤、放射性医薬製剤、ホルモン、鎮痛剤、非ステロイド系抗炎症剤、麻酔剤、鎮静剤、神経筋遮断剤、抗菌剤、抗寄生虫剤、抗ウイルス剤、インターフェロン、硝酸塩、 ざ瘡製剤、アンドロゲン剤、抗糖尿病剤、抗痛風剤、抗ヒスタミン剤、鎮咳剤、鬱血除去剤、去痰剤、抗潰瘍剤、下剤、抗凝血剤、免疫化剤、抗痙攣剤、抗パーキンソン剤、エストロゲン様製剤、甲状腺製剤、又は鉄含有抗貧血剤であることを特徴とする請求項２６に記載の方法。
【請求項２８】
前記非気体性音響画像化物質は、更に、磁気共鳴画像化物質、電子スピン共鳴画像化物質、分光画像化物質、陽電子放射断層撮影用画像化物質、光学画像化物質、Ｘ線画像化物質、核医学用画像化物質、又はこれらの組み合わせを含むこと
を特徴とする請求項１９に記載の方法。
【請求項２９】
前記分光画像化物質は、核磁気共鳴分光画像化物質又はラマン分光画像化物質を含むことを特徴とする請求項２８に記載の方法。
【請求項３０】
前記非気体性音響画像化物質は、常磁性又は超常磁性素子、放射性核種、又は光活性剤を含むことを特徴とする請求項２９に記載の方法。
【請求項３１】
前記温度の変化は、前記結合した非気体性音響画像化物質を活性化して、該結合非気体性音響画像化物質の温度を上昇し、かつその表面の音響反射率を増加することを含むことを特徴とする請求項１９に記載の方法。
【請求項３２】
前記活性化は、超音波、短波、マイクロ波、磁気照射、電磁エネルギー、又はこれらの組み合わせからのエネルギーを生成することによって行なわれることを特徴とする請求項３１に記載の方法。
【請求項３３】
前記結合非気体性音響画像化物質の温度の変化は、該結合非気体性音響画像化物質の温度を低下して、前記超音波ターゲットの音響反射率の測定可能な低下を引き起こすことを含むこと
を特徴とする請求項１９に記載の方法。
【請求項３４】
前記結合非気体性音響画像化物質の温度の低下は、寒冷療法又は心臓バイパス形成手術の一環として実行されることを特徴とする請求項３３に記載の方法。
【請求項３５】
前記温度の変化は、少なくとも５℃毎の前記結合非気体性音響画像化物質の温度の変化を含むことを特徴とする請求項１９に記載の方法。
【請求項３６】
（１）組織に結合しかつ温度の変化に応じて音響反射率の変化を引き起こす非気体性音響画像化物質を患者に投与すること、
（２）前記組織に結合した前記非気体性音響画像化物質の音響反射率を検出すること、及び
（３）前記組織に結合した前記非気体性音響画像化物質の温度を計算すること
を含むことを特徴とする患者の組織の温度モニター方法。
【請求項３７】
前記方法は、更に、前記組織及び該組織に結合した前記非気体性音響画像化物質の温度を変化することを含むこと、及び
前記検出は、前記組織に結合した前記非気体性音響画像化物質の音響反射率の変化の検出を含むことを特徴とする請求項３６に記載の方法。

【請求項３８】
前記非気体性音響画像化物質は、液体フルオロカーボンを含むエマルジョンから構成されることを特徴とする請求項３６に記載の方法。
【請求項３９】
前記フルオロカーボンは、ペルフルオロオクタンであることを特徴とする請求項３８に記載の方法。
【請求項４０】
前記非気体性音響画像化物質は、前記組織に結合するリガンドを有することを特徴とする請求項３６に記載の方法。
【請求項４１】
前記リガンドは、抗体、抗体のフラグメント、ポリペプチド、ペプチド様物質、多糖、アプタマー、脂質、核酸、又はレクチンであることを特徴とする請求項４０に記載の方法。
【請求項４２】
前記非気体性音響画像化物質は、ビオチン剤と結合したリガンド、ビオチン剤と結合したエマルジョン、及び該ビオチン結合リガンド及び該ビオチン結合エマルジョンに固定化されたアビジン剤を含むことを特徴とする請求項４１に記載の方法。
【請求項４３】
前記非気体性音響画像化物質は、エマルジョンに固定化されたプライマー物質に固定化されたリガンドを含むことを特徴とする請求項４１に記載の方法。
【請求項４４】
前記エマルジョンは、更に、生理活性剤を含むことを特徴とする請求項３６に記載の方法。
【請求項４５】
前記生理活性剤は、抗悪性腫瘍剤、放射性医薬製剤、ホルモン、鎮痛剤、非ステロイド系抗炎症剤、麻酔剤、鎮静剤、神経筋遮断剤、抗菌剤、抗寄生虫剤、抗ウイルス剤、インターフェロン、硝酸塩、 ざ瘡製剤、アンドロゲン剤、抗糖尿病剤、抗痛風剤、抗ヒスタミン剤、鎮咳剤、鬱血除去剤、去痰剤、抗潰瘍剤、下剤、抗凝血剤、免疫化剤、抗痙攣剤、抗パーキンソン剤、エストロゲン様製剤、甲状腺製剤、又は鉄含有抗貧血剤であることを特徴とする請求項４４に記載の方法。
【請求項４６】
前記非気体性音響画像化物質は、更に、磁気共鳴画像化物質、電子スピン共鳴画像化物質、分光画像化物質、陽電子放射断層撮影用画像化物質、光学画像化物質、Ｘ線画像化物質、核医学用画像化物質、又はこれらの組み合わせを含むことを特徴とする請求項３６に記載の方法。
【請求項４７】
前記分光画像化物質は、核磁気共鳴分光画像化物質又はラマン分光画像化物質を含むことを特徴とする請求項４６に記載の方法。
【請求項４８】
前記非気体性音響画像化物質は、常磁性又は超常磁性素子、放射性核種、又は光活性剤を含むことを特徴とする請求項４７に記載の方法。
【請求項４９】
前記温度の変化は、前記結合した非気体性音響画像化物質を活性化して、該結合非気体性音響画像化物質の温度を上昇し、かつその表面の音響反射率を増加することを含むことを特徴とする請求項３６に記載の方法。
【請求項５０】
前記活性化は、超音波、短波、マイクロ波、磁気照射、電磁エネルギー、又はこれらの組み合わせからのエネルギーを生成することによって行なわれることを特徴とする請求項４９に記載の方法。
【請求項５１】
前記結合非気体性音響画像化物質の温度の変化は、前記組織の音響反射率の測定可能な低

下を引き起こすために、該結合非気体性音響画像化物質の温度を低下することを含むことを特徴とする請求項３６に記載の方法。
【請求項５２】
前記結合非気体性音響画像化物質の温度の低下は、寒冷療法又は心臓バイパス形成手術の一環として実行されることを特徴とする請求項５１に記載の方法。
【請求項５３】
前記温度の変化は、少なくとも５℃毎の前記結合非気体性音響画像化物質の温度の変化を含むことを特徴とする請求項３６に記載の方法。
【請求項５４】
ターゲットと結合する温度感受性音響画像化物質を有し、ターゲットの温度変化を測定する測定装置において、該装置は、
前記温度感受性音響画像化物質の温度を変化するよう構成される要素、
前記ターゲットに音響エネルギーを送達するよう構成される超音波源、
その表面の音響反射率を測定するよう構成される超音波検出要素、及び
温度変化がない場合の前記ターゲットの音響反射率に対する、温度変化の際の前記ターゲットの音響反射率を決定するコンパレータ
を有することを特徴とする測定装置。
【請求項５５】
前記コンパレータは、前記ターゲットに結合した前記温度感受性音響画像化物質の温度変化の前後における該ターゲットの音響反射率の差を決定すること
を特徴とする請求項５４に記載の装置。
【請求項５６】
前記コンパレータは、前記ターゲットに結合した前記温度感受性音響画像化物質の変化される温度が低下された後の該ターゲットの音響反射率に対する、該ターゲットに結合した該温度感受性音響画像化物質の温度変化の際の該ターゲットの音響反射率の差を決定することを特徴とする請求項５５に記載の装置。
【請求項５７】
前記超音波源及び前記超音波画像化要素は、少なくとも１つの超音波トランスデューサを有することを特徴とする請求項５４に記載の装置。
【請求項５８】
前記温度感受性音響画像化物質の温度を変化するよう構成される要素は、エネルギー源を有することを特徴とする請求項５４に記載の装置。
【請求項５９】
前記エネルギー源は、超音波、短波、マイクロ波、磁気照射、電磁エネルギー、又はそれらの組み合わせを生成することを特徴とする請求項５８に記載の装置。
【請求項６０】
前記エネルギー源は、０．１Ｗ／ｃｍ^{2}より大きく２０００Ｗ／ｃｍ^{2}より小さい強度を有する超音波エネルギー源であることを特徴とする請求項５９に記載の装置。
【請求項６１】
前記温度感受性音響画像化物質の温度を変化するよう構成される要素は、エネルギーアブソーバーであることを特徴とする請求項５４に記載の装置。
【請求項６２】
前記エネルギーアブソーバーは、低温プローブを有することを特徴とする請求項６１に記載の装置。
【請求項６３】
前記温度感受性音響画像化物質の温度を変化するよう構成される要素は、少なくとも５℃毎に前記結合した温度感受性音響画像化物質の温度を変化するよう構成されること
を特徴とする請求項５４に記載の装置。
【請求項６４】
前記コンパレータは、前記温度感受性音響画像化物質の温度の上昇前後におけるターゲッ

ト表面の音響反射率の差を含む画像を生成するよう構成されること
を特徴とする請求項５４に記載の装置。
【請求項６５】
前記コンパレータは、前記温度感受性音響画像化物質の温度の上昇前後におけるターゲット表面の音響反射率の差を含むカラー画像を生成するよう構成されること
を特徴とする請求項６４に記載の装置。
【請求項６６】
磁気共鳴映像法、電子スピン共鳴映像法、分光映像法、陽電子放射断層撮影法、光学的映像法、Ｘ線映像法、核医学映像法、又はこれらの組み合わせを実行するよう構成される要素を更に有すること
を特徴とする請求項５４に記載の装置。
【請求項６７】
前記分光映像法は、核磁気共鳴分光映像法又はラマン分光映像法を含むこと
を特徴とする請求項６６に記載の装置。
【発明の詳細な説明】
【技術分野】
【０００１】
本発明は、一般に、超音波検出及び画像法（イメージング）に関し、より好ましくは、ターゲットに結合した温度依存性造影剤の温度の変化に基づき超音波反射率の変化を検出するための新規な組成物、方法及び装置に関する。
【背景技術】
【０００２】
分子映像法（分子イメージング）は、部位標的造影剤を用いた組織中の分子マーカーの特異的検出を可能にすることによって、伝統的な臨床的映像法の有用性を向上することができる（Weissleder、Radiology、1999年、212巻、609-614頁）。部位標的超音波（造影）剤に対する３つのアプローチが報告されているが、これらは、リポソームの使用（Alkan-Onyukselら、Ｊ. Pharm. Sci、1996年、85巻、486-490頁；Demosら、J. Pharm. Sci.、1997年、86巻、167-171頁；Demosら、J. Am. Col. Cardiol.、1999年、33巻、867-875頁）、微小気泡の使用（Mattreyら、Am. J. Cardiol.、1984年、54巻、206-210頁；Ungerら、Am. J. Cardiol.、1998年、81巻、58G-61G；Villanuevaら、Circulation、1998年、98巻、1-5頁；Klibanovら、Acad. Radiol.、1998年、5巻、S243-S246頁）又はナノエマルジョンの使用（Lanzaら、Circulation、1996年、94巻、3334-3340頁；Lanzaら、J. Acoust. Soc. Am.、1998年、104巻、3665-3672頁；Lanzaら、Ultrasound Med. Biol.、1997年、23巻、863-870頁）に基づいている。リポソームは、水中においてリン脂質により自然に形成される球形の二重膜小胞である。脱水－再水和プロセスにより生成される多層脂質二分子膜は、リポソームの内部において内部小胞を形成し、音響反射率を増加させることができる（Alkan-Onyukselら、1996年、上記；Demosら、1997年、上記；Demosら、1999年、上記）。第２のアプローチでは、その灌流適用に加えて部位標的様式のために、微小気泡が提案された。微小気泡は、血栓（Ungerら、1998年、上記；Lanzaら、Ultrasound. Med. Biol.、1997年、23巻、863-870頁）、アビジン被覆ペトリ皿（Klibanovら、1998年、上記）及び活性化内皮細胞（Villanueva et al., 1998, supra）を標的としていた。血栓と部位標的剤との相互作用の試験を行なった研究者もいた。とりわけ、アンガー等は、in vitro及びin vivoの何れにおいてもMRX-408（気泡ベースの造影剤）が首尾良く結合したのを観察した（Ungerら、1998年、上記）。
【０００３】
部位標的ナノエマルジョンは、脂質被包性液体ペルフルオロカーボンナノパーティクルからなる非気体音響造影剤である(Lanzaら、米国特許第5,690,907号;同第5,780,010号;及び同第5,958,371号参照)。ナノパーティクルは、直径約２５０ｎｍである。ペルフルオロカーボンナノパーティクルエマルジョンは、in vitro及びin vivoの血栓調製物を標的とした場合、かなり大きい音響的コントラストを示した（Lanzaら、1998年、上記；Lanzaら、

1997年、上記)。
【０００４】
部位標的造影剤の使用に際して直面する問題の１つとして、周囲の柔組織からの該パーティクルの高感度検出及び区別化がある。標的基質自体が音響発生性を有し又は当該基質表面からの信号が少々視界又は角度依存性でありうるので、血管表面上又はその近傍における病理学的変化の検出には障害が生じることがある。このような問題を解決しようとするなかで画像化技術が発達した。柔組織からの血流中の微小気泡の区別化を可能とするために第二高調波又は高調波及びパワー高調波ドップラーイメージングが使用された（Burnsら、Clinical Radiol.、1996年、51巻、50-55頁；Kasprzakら、Am. J. Cardiol.、1999年、83巻、211-217頁；Seniorら、Am. Heart J.、2000年、139巻、245-251頁；Spencerら、J. Am. Soc. Echo.、2000年、13巻、131-138頁参照）。しかしながら、柔組織もまた、第二高調波の後方散乱信号を発生する。更に、造影剤は、ドップラー技術の感度に対して余りにも遅い速度を示すことがある。微小気泡の後方散乱断面の増加の原因である共鳴現象とは異なり、部位標的ナノパーティクルエマルジョンからの反射増強を大きくする機構は、パーティクルが結合する表面における音響インピーダンスのミスマッチが原因であると報告されている（Lanzaら、1998年、上記）。それゆえ、部位標的音響造影剤、及びとりわけナノエマルジョン造影剤が造影剤として使用するにもかかわらず、より大きいコントラストを生成する手法を開発することにより、高感度の超音波分子イメージングシステムを実現できるかもしれない。
【０００５】
低速で超音波を伝送するペルフルオロカーボン液（複数）が知られている（Lagemannら、J. Am. Chem Soc.、1948年、70巻、2994-2996頁；Gupta、 Acustica、1979年、42巻、 273-277頁）。これらの物質を通る超音波の小さな速度は、温度上昇と共に直線的に超音波の速度が低下する温度依存性であることが示された（Narayanaら、Acoustics Letters、1986年、9巻、137-143頁）。この観察結果により音響レンズ（Id.）が開発可能であることが報告された。それにもかかわらず、ペルフルオロカーボン液内での超音波の速度の温度依存性の、超音波イメージングシステムへの適用可能性については、未だ提案されていない。
【０００６】
超音波エネルギーは、上述のような超音波イメージング法において部位標的造影剤へと適用された。この以前の仕事の多くは分子イメージングに注がれており、そのため低レベルの超音波エネルギーしか使用されず、標的とされた表面の温度変化が起こることについては報告されなかった。微小気泡超音波イメージングシステムでは、気体の微小気泡を形成するために液体前駆物質に十分なエネルギーが加えられた（Lohrmannら、米国特許第5,536,489号；Unger, 米国特許第5,542,935号）。提案された１つの手法によれば、相変化をin vivoで引き起こすためにエネルギーが加えられる。そのような手法では、温度変化は、気体状前駆体を気体状微小気泡へ変化させることに使用されるものであり、これら以前の研究はいずれも、液体状態を維持する超音波造影剤の温度を変化させること、又は超音波検出を向上するための基礎としての非気体性造影剤の温度の変化を利用することについては開示も示唆もしていない。
【発明の開示】
【発明が解決しようとする課題】
【０００７】
従って、コントラストを向上しかつ超音波分子イメージングシステムの感度を大きくする手法の開発に対する必要性が引き続き存在する。
【課題を解決するための手段】
【０００８】
それゆえ、本発明者らは、この課題解決のために、非気体状フルオロカーボン液を含みかつ標的（ターゲット）に結合するナノパーティクルの温度の変化が、該ターゲットの音響反射率に検出可能な変化を引き起こすということを首尾よく発見した。当該ターゲットに

隣接するがナノパーティクルによって結合されない非標的領域は、音響反射率の変化をほとんど示さないか、検出可能なほどには示さない。その結果、部位標的ナノパーティクルの音響反射率の温度依存性の変化により、超音波反射（率）の感度の大きな測定、及びコントラストの大きなイメージング（画像化）が可能となる。

【０００９】

本発明の一実施形態では、超音波ターゲットの音響反射率の変化方法が提供される。この方法は、（１）当該超音波ターゲットに結合しかつ温度の変化に応じて音響反射率の変化を引き起こす非気体性音響画像化（造影）物質を該超音波ターゲットに投与すること、及び（２）前記超音波ターゲットと結合した前記非気体性音響画像化物質の音響反射率に測定可能な変化を引き起こすよう温度を変化させること、を特徴とする。非気体性音響画像化物質は、好ましくは、液体フルオロカーボンを含むナノパーティクルエマルジョンから構成される。非気体性音響画像化物質は、好ましくは、超音波ターゲットと結合するリガンドを含む。

【００１０】

本発明の他の一実施形態では、超音波ターゲットの増強された音響反射率の測定方法が提供される。この方法は、（１）当該超音波ターゲットに結合しかつ温度の変化に応じて音響反射率の変化を引き起こす非気体性音響画像化（造影）物質を該超音波ターゲットに投与すること、（２）前記超音波ターゲットと結合した前記非気体性音響画像化物質の音響反射率に測定可能な変化を引き起こすよう温度を変化させること、及び（３）前記結合した非気体性音響画像化物質の音響反射率の変化を検出すること、を特徴とする。音響反射率の変化の検出は、好ましくは、（ａ）前記結合非気体性音響画像化物質の温度を変化する前に反射率を測定すること、（ｂ）前記結合非気体性音響画像化物質の温度を変化した後に反射率を測定すること、及び（ｃ）前記結合非気体性音響造影物質の温度を変化する前の反射率に対する前記結合非気体性音響画像化物質の温度を変化した後の反射率の変化を決定すること、を含む。

【００１１】

本発明の他の一実施形態では、患者の組織の温度のモニター方法が提供される。該方法は、（１）前記組織に結合しかつ温度の変化に応じて音響反射率の変化を引き起こす非気体性音響画像化（造影）物質を該患者に投与すること、（２）前記組織に結合した前記非気体性音響画像化物質の音響反射率を検出すること、及び（３）前記組織に結合した前記非気体性音響画像化物質の温度を計算することを特徴とする。この方法では、好ましくは、温度の変化をモニターし、更に、この方法では、前記組織及び該組織に結合した前記非気体性音響画像化物質の温度を変化すること、を含む。音響反射率の検出は、前記組織に結合した前記非気体性音響画像化物質の音響反射率の変化の検出を含む。

【００１２】

本発明の一側面によれば、温度の変化は、結合した非気体性ナノパーティクルを活性化して該結合物質の温度を上昇しかつ該ターゲットの音響反射率を増強することによって惹起可能である。ナノパーティクルは、超音波、短波、マイクロ波、磁気照射、電磁エネルギー、又はこれらの組み合わせによって活性化可能である。

【００１３】

本発明の他の一側面によれば、前記ターゲットの音響反射率の測定可能な低下を引き起こすために、結合ナノパーティクルの温度を低下することができる。

【００１４】

本発明の方法は、ナノパーティクルに組み込まれる生理活性剤と一緒の投与と共に使用することができる。更に、例えば磁気共鳴映像法、電子スピン共鳴映像法、分光（スペクトロスコピック）映像法、陽電子放射断層撮影法、光学的映像法、Ｘ線映像法、核医学映像法、又はこれらの組み合わせのような他のイメージング技術の使用に好適な１又は２以上の画像化物質をナノパーティクルに組み込むことにより音響イメージングと共に他のイメージング技術を使用することも可能である。

【００１５】

本発明の他の一実施形態によれば、ターゲットと結合する温度感受性音響画像化（造影）物質を有し、ターゲットの温度変化を測定する測定装置が提供される。該装置は、前記温度感受性音響画像化物質の温度を変化するよう構成される要素、前記ターゲットに音響エネルギーを送達するよう構成される超音波源、その表面の音響反射率を測定するよう構成される超音波検出要素、及び温度変化がない場合の前記ターゲットの音響反射率に対する、温度変化の際の前記ターゲットの音響反射率を決定するコンパレータ（比較測定器）を含むことを特徴とする。１つの側面によれば、コンパレータは、前記ターゲットに結合した前記温度感受性音響画像化物質の温度変化の前後における該ターゲットの音響反射率の差を決定する。他の一側面によれば、コンパレータは、前記ターゲットに結合した前記温度感受性音響画像化物質の変化される温度が低下された後の該ターゲットの音響反射率に対する、該ターゲットに結合した該温度感受性音響画像化物質の温度変化の際の該ターゲットの音響反射率の差を決定する。温度変化要素は、前記ターゲットに結合した温度感受性音響画像化物質の温度を上昇するエネルギー源、又は前記ターゲットに結合した温度感受性音響画像化物質の温度を低下するエネルギーアブソーバーを含みうる。

【００１６】

上記の装置は、例えば、磁気共鳴映像法、電子スピン共鳴映像法、分光映像法、陽電子放射断層撮影法、光学的映像法、Ｘ線映像法、核医学映像法、又はこれらの組み合わせのような、少なくとも１つの他のイメージング（映像化）技術を実行する要素も含みうる。

【発明の効果】

【００１７】

以上より、本発明により達成される幾つかの利点の中でも、ターゲットの音響反射率を増強する方法の提供；音響的に反射性である周囲の組織から、標的組織を区別するための方法の提供；組織の温度の検出及びモニター（監視）するための方法の提供；温度変化を伴う治療上の措置中に実行するような、温度変化を検出するための方法の提供；及び上記各方法を実行するための装置の提供に注目すべきである。

【発明を実施するための最良の形態】

【００１８】

本発明によれば、超音波ターゲットの音響反射率の検出は、該超音波ターゲットに結合しかつ温度の変化と共に音響反射率に測定可能な変化を引き起こす非気体性音響画像化（造影）物質の温度を変化することによって増強され得るということが発見された。

【００１９】

音響画像化物質は、好ましくは、先に記載された（米国特許第5,780,010号、同第5,958,371号、及び同第5,989,520号参照）ようなナノパーティクルエマルジョンである。本発明のナノパーティクルエマルジョンは、完全に分散される少なくとも２つの不混和性液体、好ましくは例えば油のような水中で分散される疎水性材料、から構成される。エマルジョンは、典型的には直径が０．１μｍより大きい小滴又はナノパーティクルの形態をとる。エマルジョンナノパーティクルの安定性を高めるため、界面活性剤又は微細粉末（finely-divided solids）のような添加物をエマルジョンナノパーティクルに組み込むこともできる。

【００２０】

水中油型エマルジョンの油相は、エマルジョンの質量に関し、好ましくは５～５０質量％、より好ましくは２０～４０質量％である。油又は疎水成分は、温度の変化（即ち、プラス又はマイナス方向への変化）と共に変化する音響インピーダンスを示し、好ましくはフルオロケミカル液体である。これらのフルオロケミカルは、直鎖状、有枝鎖状、及び環状ペル（過）フルオロカーボン、直鎖状、有枝鎖状、及び環状ペルフルオロ第三アミン、直鎖状、有枝鎖状、及び環状ペルフルオロエーテル及びチオエーテル、クロロフルオロカーボン、及びペルフルオロエーテル重合体等を含む。５０％まで水素が置換された化合物を使用することも可能であるが、ペルハロゲン（過ハロゲン）化合物が好ましい。最も好ましいのは、フッ素化（フッ素置換）化合物である。フルオロケミカル液体、即ち大気圧下、体温（例えば３７℃）以上の温度で液体である物質、であればなんでも、本発明のフル

オロケミカルエマルジョンを調製するために使用することができる。しかしながら、多くの目的に供するためには、安定性がより高められたエマルジョンフルオロケミカルが好ましい。そのようなエマルジョンを得るためには、５０℃より大きい沸点を有するフルオロケミカル液体が好ましく、最も好ましくは凡そ８０℃より大きい沸点を有するフルオロケミカル液体である。指針をなす決定的要因は、熱誘導及び画像化を意図した条件下において、例えばフルオロケミカル等の油が液相中に残存する（気体への転化は１０％未満）ようにすることであろう。

【００２１】

本発明のナノパーティクルエマルジョンについて、本書において用語「非気体性」又は「液体」について言及する場合、ナノパーティクルの内部体積の１０％未満が、ナノパーティクルの全体積に対し（即ち、ｖ／ｖで）気相をなすことを意味するよう意図しているが、この値は、好ましくは凡そ８％（ｖ／ｖ）以下、より好ましくは５％（ｖ／ｖ）以下、最も好ましくは２％（ｖ／ｖ）以下である。ここで使用した用語「凡そ」は、表示した数値の前後の値（１０％）の範囲を含むことを意図している。例えば、凡そ８％とは、７．２％～８．８％の範囲の値を含むことを意図している。

【００２２】

本発明のナノパーティクルエマルジョンは、脂質でカプセル被膜化されていると好ましい。特別な実施形態では、脂質カプセル被膜化パーティクルは、ペルフルオロカーボンから構成されてもよいが、このエマルジョンパーティクルは、誘導体化された天然又は合成リン脂質、脂肪酸、コレステロール、脂質、スフィンゴミエリン、トコフェロール、糖脂質、ステリルアミン、カルジオリピン、エーテル又はエステル結合した脂肪酸を有する脂質、又は脂質重合体からなる外膜を有する。

【００２３】

フルオロカーボンエマルジョン、とりわけペルフルオロカーボンエマルジョンは、生物医学的分野への適用に好適であり、本発明のパーティクルでの使用にも好適である。ペルフルオロカーボンエマルジョンは、安定で、生物学的に不活性で、主として経肺胞蒸発（trans-pulmonic alveolae evaporation）によって容易に代謝されることが知られている。更に、その粒子サイズは小さいため、経肺通路（transpulmonic passage）に容易に適応し、かつその循環の半減期（４～８時間）は、他の剤の循環半減期よりも大きいので有利である。また、ペルフルオロカーボンは、人工血液代用剤としての使用を含む、広範囲に亘る生物医学分野への適用にこれまで使用されてきた。本発明で使用するために、フルオロカーボンがフルオロカーボン－ハイドロカーボン、ペルフルオロアルキル化エーテル、ポリエーテル又はクラウンエーテルであるようなフルオロカーボンエマルジョンを含む複数のフルオロカーボンエマルジョンを使用してもよい。有用なペルフルオロカーボンエマルジョンは、米国特許第4,927,623号、同第5,077,036号、同第5,114,703号、同第5,171,755号、同第5,304,325号、同第5,350,571号、同第5,393,524号、及び同第5,403,575号に記載されており、ペルフルオロカーボン化合物が、ペルフルオロトリブチルアミン、ペルフルオロデカリン、ペルフルオロオクチルブロマイド、ペルフルオロジクロロオクタン、ペルフルオロデカン、ペルフルオロトリプロピルアミン、ペルフルオロトリメチルシクロヘキサン、又は他のペルフルオロカーボン化合物であるようなペルフルオロカーボンエマルジョンを含む。更に、そのようなペルフルオロカーボン化合物の混合物を、本発明の実施に使用されるエマルジョンに組み込んでもよい。

【００２４】

本発明に有用なペルフルオロカーボンエマルジョンの具体的な実施形態として、ペルフルオロジクロロオクタンエマルジョンを挙げることができるが、これは、その脂質の被膜が、約５０～約９９．５モルパーセントのレシチン、好ましくは約５５～約７０モルパーセントのレシチン、０～５０モルパーセントのコレステロール、好ましくは約２５～約４５モルパーセントのコレステロール、及び約０．５～約１０モルパーセントのビオチン化ホスファチジルエタノールアミン、好ましくは約１～約５モルパーセントのビオチン化ホスファチジルエタノールアミンを含む。ホスファチジルセリンのような他のリン脂質をビオ

チン化し、ステアリルアミンのような脂肪族アシル基をビオチンと複合化し、又はコレステロール若しくは他の脂溶性化合物をビオチン化して、脂質でカプセル被膜化されるパーティクルのための脂質の被膜に組み込んでもよい。本発明の実施に使用されるビオチン化ペルフルオロカーボンの例の調製については、以下において説明するが、これは既知の手順に従っている。

【００２５】

脂質カプセル被膜化パーティクルがエマルジョンではなくリポソームによって構成される場合、そのようなリポソームは、文献（例えば、Kimelbergら、CRC Crit. Rev. Toxicol.、1978年、6巻、25頁、；Yatvinら、Medical Physics、1982年、9巻、149頁参照）に一般的に記載されているような方法で調製することができる。リポソームは、当業者には既知であり、一般的に、レシチン及びステロール、卵ホスファチジルコリン、卵ホスファチジン酸、コレステロール及びα－トコフェロールを含む脂質材料から構成される。

【００２６】

例えば界面活性剤等のエマルジョン形成剤は、エマルジョンの形成を容易にしかつその安定性を向上するために使用することができる。フルオロケミカル液体のエマルジョンの形成を容易にするために、典型的には、水相界面活性剤が使用されてきた。界面活性剤は、親水性部分と疎水性部分を両方とも含む物質であればどんな物質でもよい。水又は溶媒に添加されると、界面活性剤は、表面張力を低減する。好ましい界面活性剤は、リン脂質とコレステロールである。

【００２７】

何れかの又は種々（複数）の脂質系界面活性剤を脂質単分子膜に組み込んでもよい。当該界面活性剤は、好ましくは天然又は合成のリン脂質であるが、脂肪酸、コレステロール、リゾリピド、スフィンゴミエリン、トコフェロール、糖脂質、ステアリルアミン、カルジオリピン、 プラスマロゲン、エーテル又はエステル結合した脂肪酸を有する脂質、脂質重合体、及び脂質と複合化したポリエチレングリコールでもあってもよい。他の既知の界面活性剤添加物、例えば、ＰＬＵＲＯＮＩＣ Ｆ－６８、ＨＡＭＰＯＳＹＬ Ｌ３０（W. R. Grace Co., Nashua, N.H.）、ドデシル硫酸ナトリウム、Ａｅｒｏｓｏｌ ４１３（American Cyanamid Co., Wayne, N.J.）、Ａｅｒｏｓｏｌ ２００（American Cyanamid Co.）、ＬＩＰＯＰＲＯＴＥＯＬ ＬＣＯ（Rhodia Inc., Mammoth, N.J.）、ＳＴＡＮＤＡＰＯＬ ＳＨ １３５（Henkel Corp., Teaneck, N.J.）、ＦＩＺＵＬ １０－１２７（Finetex Inc., Elmwood Park, N.J.）、及びＣＹＣＬＯＰＯＬ ＳＢＦＡ ３０（Cyclo Chemicals Corp., Miami, Fla.）;以下の商標名で販売されているような両性界面活性剤：Ｄｅｒｉｐｈａｔ（商標） １７０（Henkel Corp.）、ＬＯＮＺＡＩＮＥ ＪＳ（Lonza, Inc.）、ＮＩＲＮＯＬ Ｃ２Ｎ－ＳＦ（Miranol Chemical Co., Inc., Dayton, N.J.）、ＡＭＰＨＯＴＥＲＧＥ Ｗ２（Lonza, Inc.）、及びＡＭＰＨＯＴＥＲＧＥ ２ＷＡＳ（Lonza, Inc.）;以下の商標名で販売されているような非イオン界面活性剤：ＰＬＵＲＯＮＩＣ Ｆ－６８（BASF Wyandotte, Wyandotte, Mich.）、ＰＬＵＲＯＮＩＣ Ｆ－１２７（BASF Wyandotte）、ＢＲＩＪ ３５（ICI Americas; Wilmington, Del.）、ＴＲＩＴＯＮ Ｘ－１００（Rohm and Haas Co., Philadelphia, Pa.）、ＢＲＩＪ ５２（ICI Americas）、ＳＰＡＮ ２０（ICI Americas）、ＧＥＮＥＲＯＬ １２２ ＥＳ（Henkel Corp.）、ＴＲＩＴＯＮ Ｎ－４２（Rohm and Haas Co.,）、Ｔｒｉｔｏｎ（商標） Ｎ－１０１（Rohm and Haas Co.,）、ＴＲＩＴＯＮ Ｘ－４０５（Rohm and tlaas Co.,）、ＴＷＥＥＮ ８０（ICI Americas）、ＴＷＥＥＮ ８５（ICI Americas）、及びＢＲＩＪ ５６（ICI Americas）等を、エマルジョンの安定化向上を促進するために、０．１０～５．０質量％の濃度で、単独又は組み合わせて使用してもよい。

【００２８】

乳化されるべきフルオロケミカル液体に可溶なフルオロ化界面活性剤も使用することができる。好適なフルオロケミカル界面活性剤は、ペルフルオロヘキサン酸及びペルフルオロオクタン酸のようなフルオロ化アルカン酸、及びアミドアミン誘導体を含む。これら界面活性剤は、一般的に、０．０１～５．０質量％の濃度、好ましくは０．１～１．０質量％

の濃度で使用される。他の好適なフルオロケミカル界面活性剤は、ペルフルオロ化アルコールリン酸エステル及びそれらの塩、ペルフルオロ化スルホンアミドアルコールリン酸エステル及びそれらの塩、ペルフルオロ化アルキルスルホンアミドアルキレン第４アンモニウム塩、Ｎ，Ｎ－（カルボキシル置換低級アルキル）ペルフルオロ化アルキルスルホンアミド、及びそれらの混合物を含む。ここで用いた用語「ペルフルオロ化」は、界面活性剤が少なくとも１つのペルフルオロ化されたアルキル基を含むということを意味するものとする。

【００２９】

好適なペルフルオロ化アルコールリン酸エステルは、モノ－及びビス（１Ｈ，１Ｈ，２Ｈ，２Ｈ－ペルフルオロアルキル）リン酸塩（エステル）のジエタノールアミン塩の遊離酸を含む。このようなリン酸塩は、商標名ＺＯＮＹＬ ＲＰ（E. I. Dupont de Nemours and Co., Wilmington, Del.）のものが入手可能であるが、既知の方法によって分解されて対応する遊離酸を生成する。好適なペルフルオロ化スルホンアミドアルコールリン酸エステルは、米国特許第3,094,547号に記載されている。好適なペルフルオロ化スルホンアミドアルコールリン酸エステル及びそれらの塩は、ペルフルオロ－ｎ－オクチル－Ｎ－エチルスルホンアミドエチルリン酸エステル、ビス（ペルフルオロ－ｎ－オクチル－Ｎ－エチルスルホンアミドエチル）リン酸エステル；ビス（ペルフルオロ－ｎ－オクチル－Ｎ－エチルスルホンアミドエチル）リン酸、ビス（ペルフルオロデシル－Ｎ－エチルスルホンアミドエチル）リン酸、及びビス（ペルフルオロヘキシル－Ｎ－エチルスルホンアミドエチル）リン酸のアンモニウム塩を含む。好ましい処方物は、ホスファチジルコリン、誘導ホスファチジルエタノールアミン、及びコレステロールを水性界面活性剤として使用する。

【００３０】

脂質カプセル被膜化エマルジョンには、核酸材料がパーティクル表面に付着するのを容易にするカチオン性脂質を界面活性剤（含有）層に処方してもよい。カチオン性脂質は、１，２－ジアシル－３－トリメチルアンモニウムプロパン（ＴＡＰ），１，２－ジアシル－３－ジメチルアンモニウムプロパン（ＤＡＰ），ＤＣ－コレステロール（ＤＣ－Ｃｈｏｌ），ジメチルジオクタデシルアンモニウムブロマイド（ＤＤＡＢ），１，２－ジアシル－ｓｎ－グリセロ－３－エチルホスホコリン ＤＯＴＭＡ，Ｎ－［１－（２，３－ジオレオイロキシ）プロピル］－Ｎ，Ｎ，Ｎ－トリメチルアンモニウムクロライド；ＤＯＴＡＰ，１，２－ジオレオイロキシ－３－（トリメチルアンモニウム）プロパン；及びＤＯＴＢ，１，２－ジオレオイル－３－（４’－トリメチル－アンモニウム）ブタノイル－ｓｎ－グリセロールを含むがこれらに限定されない。一般に、脂質界面活性剤単分子膜中におけるカチオン性脂質と非カチオン性脂質のモル比は、例えば、１：１０００～２：１であり、好ましくは２：１～１：１０、より好ましくは１：１～１：２．５、最も好ましくは１：１である（カチオン性脂質のモル数と非カチオン性脂質、例えばＤＰＰＣ、のモル数の比）。さまざまな脂質が、エマルジョン界面活性剤の非カチオン性脂質成分を構成することができる。上述の化合物に加え、とりわけジパルミトイルホスファチジルコリン、ジパルミトイルホスファチジル－エタノールアミン又はジオレオイルホスファチジルエタノールアミンがこれに該当する。上述したカチオン性脂質の代わりに、ポリリジン又はポリアルギニンのようなカチオン性ポリマーを有する脂質も脂質界面活性剤に含まれうるが、これらは、例えば遺伝子材料又はそのアナログのような負に帯電した薬剤をエマルジョンパーティクルの外側に結合させてもよい。

【００３１】

本発明の音響造影（画像化）物質は、少なくとも１つのペルフルオロカーボンから構成されることが好ましいが、ターゲットに結合すると温度依存性の反射率を示す。ペルフルオロカーボンは、温度の上昇に従って超音波の速度を直線的に減少させるということ、及び１０℃～５０℃の温度範囲に亘って密度が減少するということが報告されている（Narayanaら、1986年、上記）。それゆえ、ナノパーティクルエマルジョンの温度依存性又は例えば好ましいペルフルオロカーボン成分のようなナノパーティクルエマルジョンの構成成分の温度依存性は、エマルジョン又はその構成成分の超音波の速度を決定することによって

測定することができる。これについては以下の実施例においてより完全に説明している。この測定は、所与のペルフルオロカーボン成分に関する反射率の変化の大きさの予測に利用することができると考えられている。

【００３２】

超音波ターゲットは、in vivo又はin vitroのターゲットであってもよく、好ましくは生物学的材料であるが、該ターゲットは生物学的材料である必要はない。ターゲットは、音響造影物質が結合する表面、又は音響造影物質が侵入しかつターゲットの表面下の部分に結合する三次元構造体から構成されてもよい。

【００３３】

音響造影物質を超音波ターゲットに固定するために、音響造影物質にリガンドを組み込むことが好ましい。リガンドは、能動的ターゲティングを可能にするために、目的のターゲットに特異的に構成されてもよい。ここに、能動的ターゲティングとは、局在化による細胞、組織又は器官への剤のリガンド指向的部位特異的蓄積、及び細胞の表面膜又は細胞外若しくは細胞内マトリクスに現出した分子エピトープ、即ちレセプタ、脂質、ペプチド、細胞接着分子、多糖、生体高分子等への結合をいう。抗体、抗体のフラグメント、小さなオリゴペプチドのようなポリペプチド、立体構造を有する大きなポリペプチド又はタンパク質、ペプチド様物質、多糖、アプタマー、脂質、核酸、レクチン、又はこれらの組み合わせを含む様々なリガンドを使用することができる。リガンドは、細胞のエピトープ又はレセプタに特異的に結合する。

【００３４】

ここに、用語「リガンド」は、エマルジョンパーティクル自体とは異なる生物学的ターゲットの他の分子に特異的に結合する小さなターゲティング分子をいうものとする。反応には、錯体の金属原子と配位共有結合を形成するため電子対を供与又は受容する分子を必要とはしないが排除もしない。従って、リガンドは、直接的結合に対しては共有結合的に又は間接的結合に対しては非共有結合的に、音響パーティクル表面（エマルジョン）の表面に付着することができる。

【００３５】

アビジン－ビオチン相互作用は、既に多くの生物学的及び分析システムに取り入れられかつin vivoへの適用に選択されている極めて有用な非共有結合的ターゲティングシステムである。アビジンは、ビオチンに対し生理的条件下で迅速かつ安定な結合を可能にする高い親和性（10^{-15} M）を有する。この手法を利用するターゲティングシステムは、処方物に応じて、２ステップ又は３ステップで実行される。典型的には、まず、モノクローナル抗体のようなビオチン化リガンドを投与して特異的分子エピトープを「プレターゲット」する。次に、アビジンを投与して「プレターゲット」したリガンドのビオチン部分に結合させる。最後に、ビオチン化剤を添加して、アビジンに残存している空のビオチン結合部位に結合させることによって、リガンド－アビジン－エマルジョンの「サンドイッチ」を形成する。アビジン－ビオチン法は、表面抗体の存在を介する細網内皮系によるターゲット剤の加速された早期の除去（クリアランス）を回避することができる。更に、アビジンは、独立した４つのビオチン結合部位を有するが、信号の増幅を提供し、検出の感度を改善する。

【００３６】

ここに、用語「ビオチン剤」又はビオチン剤へとの結合に関する「ビオチン化」は、ビオチン、ビオサイチン、及び例えばビオチンアミドカプロン酸Ｎ－ヒドロキシスクシンイミドエステル、ビオチン４－アミドベンゼン酸、ビオチンアミドカプロイルヒドラジド及び他のビオチン誘導体及び複合体のような他のビオチン誘導体及びアナログを含むのもとする。そのほか誘導体としては、ビオチン－デキストラン、ビオチン－ジスルフィド－Ｎ－ヒドロキシスクシンイミドエステル、ビオチン－６アミドキノリン、ビオチンヒドラジド、ｄ－ビオチン－Ｎ－ヒドロキシスクシンイミドエステル、ビオチンマレイミド、ｄ－ビオチンｐ－ニトロフェニルエステル、ビオチン化ヌクレオチド、及びＮ，ε－ビオチニル－ｌ－リジンのようなビオチン化アミノ酸も含む。用語「アビジン剤」又はアビジン剤と

の結合に関する「アビジン化」は、アビジン、ストレプトアビジン、ストレプトアビジン又はアビジン複合体のようなアビジンアナログ、アビジン又はストレプトアビジンの高精製分画種、及び非アミノ酸又は部分アミノ酸変異体、組換え又は化学合成アビジンを含むものとする。

【００３７】

ターゲティングリガンドを、パーティクル表面の性質に応じ種々の方法により音響パーティクルの表面に化学的に付着させてもよい。結合は、使用するリガンドに応じてエマルジョンパーティクルが生成される前又は後に実行してもよい。リガンドのタンパク質剤への直接的化学結合では、その表面に本来的に存在する多数のアミノ基（例えばリジン）をよく利用する。或いは、ピリジルジチオプロピオネート、マレイミド又はアルデヒドのような機能的化学活性基を、パーティクルを生成した後、リガンドの結合のための化学的「フック」としてその表面に組み込むことも可能である。他の一般的な後処理法では、リガンドを付加する前に、カルボジイミドによって、表面カルボン酸塩を活性化する。選択される共有結合的連結ストラテジは、主として、リガンドの化学的性質によって決定される。モノクローナル抗体及び他の大きなタンパク質は、厳しい処理条件下では変性する場合もある；他方、炭水化物、短ペプチド、アプタマー、薬剤又はペプチド様物質の生理活性は、多くの場合保存可能である。高レベルのリガンド結合の完全性を確実にしかつターゲットパーティクルの結合活性を最大化するために、例えばポリエチレングリコール又は単純なカプロン酸エステルブリッジのようなフレキシブルなポリマースペーサアームを、活性化された表面機能性基とターゲティングリガンドの間に挿入することができる。このような付加部分は、１０ｎｍ以上とすることができ、パーティクル表面の相互作用によるリガンド結合の妨害を最小にすることができる。

【００３８】

モノクローナル抗体を、病態的分子エピトープを含む広範囲の分子エピトープの何れにも（何れかに）向けられる部位ターゲティングリガンドとして使用してもよい。既に、免疫グロブリン－γ（ＩｇＧ）クラスモノクローナル抗体を、リポソーム、エマルジョン及び他の微小気泡パーティクルに結合させて、能動的（アクティブ）部位特異的ターゲティングを提供している。これらのタンパク質は、Ｈ鎖及びＬ鎖の同一の対からなる対称的な糖タンパク質（分子量約150,000ダルトン）である。２つのアームの各々の端部の高頻度可変領域は、同一の抗原結合ドメインを提供する。可変長分枝炭水化物ドメインは、補体活性化領域に結合し、ヒンジ領域は、還元されてより小さいフラグメントを生成できるとりわけ接近容易な鎖間ジスルフィド結合を有する。

【００３９】

二価のＦ（ａｂ’）$_2$及び一価のＦ（ａｂ）フラグメントは、リガンドとして使用することができ、これらは、それぞれ、ペプシン又はパパイン消化による抗体全体の選択的開裂に由来する。Ｆｃ領域の除去により、分子の免疫原性は大きく減少し、結合糖鎖を介する非特異的肝臓取り込みは大きく減少し、かつ補体活性化及び結果としての抗体依存性細胞毒性は大きく低下する。補体固定及び関連する細胞性細胞毒性は、ターゲット部位を保存しなければならない場合は有害でありえ、又はホストキラー細胞の補充とターゲット細胞の破壊が所望の場合（例えば坑腫瘍剤）は有利でありうる。

【００４０】

モノクローナル抗体は、大抵、マウスに由来しており、程度はそれぞれ異なるが他の種に対して本来的に免疫原性である。遺伝子工学技術によるマウス抗体のヒト化により、生体適合性が改善されかつ循環半減期が長いキメラリガンドが開発された。ターゲット分子エピトープに対する組換え抗体の結合親和性は、結合イディオタイプの選択的部位特異的突然変異誘発によって改善できることがある。

【００４１】

抗体産生動物を使用することなく広範囲の異なる抗原に対する組換えヒトモノクローナル抗体フラグメントを生成するために、ファージディスプレイ技術を使用してもよい。一般に、クローニングにより、ヒトＢリンパ球の全メッセンジャーＲＮＡ（ｍＲＮＡ）から酵

素「逆転写酵素」によって演繹・合成される相補ＤＮＡ（ｃＤＮＡ）鎖の大きな遺伝子ライブラリが作られる。免疫グロブリンｃＤＮＡ鎖は、ＰＣＲ（ポリメラーゼ連鎖反応）によって増幅され、所与の抗原に特異的なＬ鎖及びＨ鎖が、ファージミドベクターに組み込まれる。このファージミドベクターを適切なバクテリアに感染させることにより、ｓｃＦｖ免疫グロブリン分子がバクテリオファージの表面に発現する。目的の抗原（例えば、タンパク質、ペプチド、核酸、及び糖）に対し免疫吸着／ファージ増殖サイクルを繰り返すことにより、特定の免疫グロブリンを発現するバクテリオファージが選択される。ターゲット抗原に厳格に特異的なバクテリオファージを適切なベクター（例えば、大腸菌、酵母、細胞）に導入し、発酵によって増殖して、天然の抗体に極めて類似する構造を有するヒト抗体フラグメントを大量に産生する。ファージディスプレイ技術により、ターゲティングと治療学的応用のための特有のリガンドの生産が可能となった。

【００４２】

ポリペプチドは、抗体のように、標的化された造影剤のためのベクター分子として使用するため大きな特異性とエピトープ親和性を有していてもよい。これらは、（例えば血小板ＧＩＩｂＩＩＩａレセプターのＲＧＤエピトープのような）特有のレセプタ配列に対し特異的な、例えば５～１０個のアミノ酸残基を有する小さいオリゴペプチドであってもよく、又はコレシストキニンのような、より大きい生理活性ホルモンであってもよい。より小さいペプチドは、ヒト化されていないマウス抗体よりも固有の免疫原性は小さいかもしれない。細胞接着分子、サイトカイン、セレクチン、カドヘドリン、Ｉｇスーパーファミリー、インテグリン等のペプチド又はペプチド（非ペプチド）アナログをターゲット治療剤送達のために使用してもよい。

【００４３】

肝細胞に局在するアシアロ糖タンパク質レセプターに対する親和性は大きいため、肝特異的に適用するためにアシアロ糖タンパク質が使用された。第一期及び第二期の肝腫瘍及び肝炎のような良性のび漫性肝臓疾患を検出するために、アシアロ糖タンパク質指向剤（主として酸化鉄と結合した磁気共鳴剤）が使用された。アシアロ糖タンパク質レセプターは、肝細胞に極めて大量に存在し（細胞当り約500,000）、急速に内部移行し、引き続き細胞表面に現れ再利用される。剤を肝ターゲットに局在化させるために、アラビノガラクタンのような多糖を使用してもよい。アラビノガラクタンは、アシアロ糖タンパク質肝レセプターに対する高親和性を呈する多末端アラビノース基を有する。

【００４４】

アプタマーは、高親和性、高特異性ＲＮＡ又はＤＮＡベースリガンドであり、in vitroでの選択実験（SELEX: systematic evolution of ligands by exponential enrichment）によって生産される。アプタマーは、２０～３０ヌクレオチドのランダム配列から作成され、分子抗原又は細胞への吸収（吸着）によって選択的にスクリーニングされ、特異的高親和性結合リガンドを生成するために濃縮される。In vivoでの安定性及び有用性を向上するために、アプタマーは、一般的に、ヌクレアーゼ消化を抑制しかつ薬物、ラベル又はパーティクルとの結合が可能となるよう化学的に修飾される。他のより単純な化学的ブリッジが、リガンド相互作用に非特異的に関与する核酸の代わりに使用されることもよくある。溶液中では、アプタマーは、立体構造が解除されているが、特異的認識を供するターゲットエピトープに絡んだり包んだりすることはできる。エピトープの周囲の核酸の特有の折り畳みにより、水素結合、静電相互作用、スタッキング、及び形状相補性を介して、差別的分子内接触が可能となる。タンパク質ベースのリガンドと比較すると、アプタマーは、安定で、熱殺菌に対しより伝導性が大きく、免疫原性は小さい。現在、アプタマーは、血管新生、活性化血小板、及び固体腫瘍を含む多数の臨床関連病態をターゲットするために使用されており、その使用は益々増えている。治療エマルジョンパーティクルのためのターゲティングリガンドとしてのアプタマーの臨床上の有効性は、核酸のリン酸基により減ぜられる負の表面電荷のクリアランス速度への影響に依存させてもよい。脂質ベースのパーティクルに関する以前の研究は、負のゼータ電位により、リポソームの循環半減期は著しく低下し、他方中性又は正のパーティクルは同様のより長い全身残存性を有するとい

うことを示唆している。
【００４５】
あるin vitroでの適用に関しMillbrathら（米国特許第5,401,634号）に記載されているような、特異的結合種をフルオロケミカル小滴に結合するための「プライマー材料」と称されているものを使用することも可能である。ここに、「プライマー材料」は、パーティクルと、ターゲティングリガンド又はターゲティングリガンドのサブユニットのようなターゲティングリガンドの成分との間で共有結合を形成するために化学的に利用可能なエマルジョン脂質界面活性剤層に組み込まれる何らかの成分又は誘導成分をいうものとする。
【００４６】
従って、特異的結合種（例えばターゲティングリガンド）を、油／水界面に直接吸着し又はプライマー材料を使用することによりカプセル被覆を行なう脂質単分子膜に固定してもよい。プライマー材料は、特異的結合又はターゲティング種と化学的に結合又は吸着するためにパーティクルに組み込まれる界面活性剤適合性化合物であれば任意のものでよい。水性連続相と、連続及び不連続相の界面でプライマー材料と吸着又は結合する生理活性リガンドとによって、エマルジョンを形成すると好ましい結果が得られる。カップリング剤及び高帯電ポリマーと特異的に反応することができるアミン、カルボキシル、メルカプト又はその他の機能性基を有する自然発生又は合成ポリマーをカップリングプロセスで使用してもよい。特異的結合種（例えば抗体）を、直接的吸着又は化学的結合によって、フルオロケミカルエマルジョンパーティクルの表面に固定してもよい。直接的吸着によって固定可能な特異的結合種の例として、小ペプチド、ペプチド様物質、又は多糖ベースの剤を挙げることができる。そのようなエマルジョンを形成するために、エマルジョンを形成する前に特異的結合種を水相に懸濁又は溶解してもよい。また、エマルジョンを形成した後特異的結合種を添加して、ｐＨ７．０のバッファ（典型的にはリン酸緩衝食塩水）中、室温（２５℃）で１．２～１８時間穏やかに振盪することによりインキュベートしてもよい。
【００４７】
特異的結合種をプライマー材料に結合すべき場合、従来のカップリング技術を利用してもよい。特異的結合種を、当業者に既知の方法により、カップリング剤を用いて、プライマー材料と共有結合的に結合してもよい。プライマー材料は、ホスファチジルエタノールアミン（ＰＥ），Ｎ－カプロイルアミン－ＰＥ，ｎ－ドデカニルアミン，ホスファチジルチオエタノール,Ｎ－１，２－ジアシル－ｓｎ－グリセロ－３－ホスホエタノールアミン－Ｎ－［４－（ｐ－マレイミドフェニル）ブチルアミド］，１，２－ジアシル－ｓｎ－グリセロ－３－ホスホエタノールアミン－Ｎ－［４－（ｐ－マレイミドメチル）シクロヘキサン－カルボキシラート］，１，２－ジアシル－ｓｎ－グリセロ－３－ホスホエタノールアミン－Ｎ－［３－（２－ピリジルジチオ）プロピオネート］，１，２－ジアシル－ｓｎ－グリセロ－３－ホスホエタノールアミン－Ｎ［ＰＤＰ（ポリエチレングリコール）2000］，Ｎ－スクシニル－ＰＥ，Ｎ－グルタリル－ＰＥ，Ｎ－ドデカニル－ＰＥ，Ｎ－ビオチニル－ＰＥ，又は Ｎ－カプロイル－ＰＥを含んでもよい。付加的なカップリング剤は、１－エチル－３－（３－Ｎ，Ｎジメチルアミノプロピル）カルボジイミドヒドロクロライド又は １－シクロヘキシル－３－（２－モルフォリノエチル）カルボジイミドメチル－ｐ－トルエンスルホネートのようなカルボジイミドを使用する。他の好適なカップリング剤には、アクロレイン、メタクロレインのようなエチレン系不飽和化合物か２－ブテナールの何れかを有するか、又はグルタルアルデヒド、プロパンジアール又はブタンジアールのような複数のアルデヒド基を有するアルデヒドカップリング剤が含まれる。カップリング剤には、ほかにも、２－イミノチオランヒドロクロライド，ジスクシンイミジルサブストレート，ジスクシンイミジルタートラート，ビス［２－（スクシンイミドオキシカルボニロキシ）エチル］スルホン，ジスクシンイミジルプロピオナート，エチレングリコールビス（スクシンイミジルサクシネート）のような二機能性Ｎ－ヒドロキシスクシンイミドエステル；Ｎ－（５－アジド－２－ニトロベンゾイロキシ）スクシンイミド，ｐ－アジドフェニルブロマイド，ｐ－アジドフェニルグリオキサル，４－フルオロ－３－ニトロフェ

ニルアジド，Ｎ－ヒドロキシスクシンイミジル－４－アジドベンゾアート，ｍ－マレイミドベンゾイル Ｎ－ヒドロキシスクシンイミドエステル，メチル－４－アジドフェニルグリオキサル，４－フルオロ－３－ニトロフェニルアジド，Ｎ－ヒドロキシスクシンイミジル－４－アジドベンゾアートヒドロクロライド，ｐ－ニトロフェニル２－ジアゾ－３，３，３－トリフルオロプロピオネート，Ｎ－スクシンイミジル－６－（４'－アジド－２'－ニトロフェニルアミノ）ヘキサノアート，スクシンイミジル４－（Ｎ－マレイミドメチル）シクロヘキサン－１－カルボキシラート，スクシンイミジル４－（ｐ－マレイミドフェニル）ブチラート，Ｎ－スクシンイミジル（４－アジドフェニルジチオ）プロピオナート，Ｎ－スクシンイミジル３－（２－ピリジルジチオ）プロピオナート，Ｎ－（４－アジドフェニルチオ）フタルアミドのようなヘテロ二機能性試薬；１，５－ジフルオロ－２，４－ジニトロベンゼン，４，４'－ジフルオロ－３，３'－ジニトロジフェニルスルホン，４，４'－ジイソチオシアノ－２，２'－ジスルホン酸スチルベン，ｐ－フェニレンジイソチオシアネート，カルボニルビス（Ｌ－メチオニンｐ－ニトロフェニルエステル），４，４'－ジチオビスフェニルアジド，エリトリトールビスカーボネートのようなホモ二機能性試薬、及びジメチルアジピミデートヒドロクロライド，ジメチルスベリミデート，ジメチル３，３'－ジチオビスプロピオンイミデートヒドロクロライドのような二機能性イミドエステル等が含まれる。特異的結合種とプライマー材料との共有結合は、上述の試薬を使用し、例えば中性のｐＨ、２５℃未満の温度の水溶液中で１時間からオーバーナイトで行なう、伝統的な既知の反応によって形成することができる。

【００４８】

本発明のエマルジョンは、種々の方法によって調製することができる。その一例として、フルオロケミカル液と、適切なプライマー材料及び／又は特異的結合種を含む水溶液との混合物を超音波処理する方法がある。一般に、これらの混合物は、界面活性剤を含む。乳化された混合物を冷却し、界面活性剤の濃度を最小化し、生理食塩水バッファで緩衝することにより、典型的には、特異的結合能の保持も、プライマー材料のカップリング能も最大化される。これらの方法により、吸収（吸着）されたプライマー材料又は特異的結合種の単位当りの活性が大きい良好なエマルジョンが得られる。

【００４９】

高濃度のプライマー材料又は特異的結合種が脂質エマルジョンに被膜形成したら、混合物を超音波処理中加熱し、該混合物が、比較的低いイオン強度と中程度から低いｐＨを持つようにする。イオン強度が低く過ぎたり、ｐＨが低く過ぎたり又は加熱し過ぎたりすると、相当分解したり、特異的結合種の有用な結合能や「プライマー材料」のカップリング能の全てが喪失したりすることもある。乳化の条件を注意深く制御・変化することにより、プライマー材料又は特異的結合種の特性を最適化しつつ、高濃度の被膜を形成することができる。

【００５０】

米国特許第4,310,505号に記載されているようにin vivoでのターゲティングのために炭水化物含有脂質を使用してもよい。この文献の記載内容は全て引照を持ってここに記載されているものとする。

【００５１】

エマルジョンを生成する他の一例として、水溶液、プライマー材料又は特異的結合種、フルオロカーボン液及び（もしあれば）界面活性剤を含む混合物の高圧流体をコントロールし、それらを互いに衝突させ、パーティクルのサイズ及び分布が制限されたエマルジョンを生成する方法がある。好ましいエマルジョンを作るために、微小流体化装置 (Microfluidics, Newton, Mass.) を使用することができる。この装置は、超音波処理又は他の伝統的な方法によってエマルジョンを後処理するためにも有用である。エマルジョン小滴の流動体を微小流体化装置に供給することにより、サイズが小さく、限定的なパーティクルサイズ分布を有する処方物が得られる。

【００５２】

乳化剤及び／又は可溶化剤をエマルジョンと共に使用してもよい。そのような剤には、ア

ラビアゴム，コレステロール，ジエタノールアミン，グリセリルモノステアレート，ラノリンアルコール，レシチン，モノ－及びジグリセリド，モノエタノールアミン，オレイン酸，オレイルアルコール，ポリオキサマー（poloxamer），ピーナッツオイル，パルミチン酸，ポリオキシエチレン５０ステアレート，ポリオキシル３５ひまし油，ポリオキシル１０オレイルエーテル，ポリオキシル２０セトステアリルエーテル，ポリオキシル４０ステアレート，ポリソルベート２０，ポリソルベート４０，ポリソルベート６０，ポリソルベート８０，プロピレングリコールジアセテート，プロピレングリコールモノステアレート，ラウリル硫酸ナトリウム，ステアリン酸ナトリウム，ソルビタンモノラウレート，ソルビタンモノオレアート，ソルビタンモノパルミテート，ソルビタンモノステアレート，ステアリン酸，トロラミン（trolamine）、及び乳化ろうが含まれるがこれらに限定されない。植物又は動物由来の脂質に見出される飽和又は不飽和炭化水素脂肪酸の代わりに脂質の成分としてフルオロ脂肪酸を有する脂質であればどれでも使用することができる。エマルジョンと共に使用可能な懸濁剤及び／又は増粘剤には、アラビアゴム，寒天，アルギン酸，モノステアリン酸アルミニウム，ベントナイト，マグマ，カルボマー９３４Ｐ，カルボキシメチルセルロース，カルシウム及びナトリウム及びナトリウム１２，カラゲーニン，セルロース，デキストリン，ゼラチン，グアーガム，ヒドロキシエチルセルロース，ヒドロキシプロピルメチルセルロース，珪酸マグネシウムアルミニウム，メチルセルロース，ペクチン，ポリエチレンオキサイド，ポリビニルアルコール，ポビドン，プロピレングリコールアルギナート，二酸化珪素，アルギン酸ナトリウム，トラガカントゴム、及びキサンガムが含まれるがこれらに限定されない。

【００５３】

有用なエマルジョンは、例えば直径約０．０１μｍ～１０μｍ、好ましくは約０．１～約０．５μｍの範囲の、広い範囲の公称パーティクル径を有しうる。乳化方法及び化学成分を変えることにより、エマルジョンパーティクルの大きさを制御・変化することができる。パーティクルの大きさは小さいものが有利であるが、それは、小さいパーティクルはより長時間循環し、かつより大きいパーティクルよりも安定性が大きい傾向があるからである。

【００５４】

標的化治療エマルジョンは、生理活性剤（例えば、薬物、プロドラッグ、遺伝子材料、放射性同位元素、又はそれらの組み合わせ）をその未変化（ネイティブ）型に組み込んだり、リガンド標的化パーティクルへの組み込み又は吸着を向上するための疎水性又は荷電成分によって修飾してもよい。生理活性剤は、プロドラッグであってもよい。プロドラッグには、Sinkylaら、J. Pharm. Sci.、1975年、64巻、181-210頁、1997年5月6日に出願された米国特許出願第08/851,780号、及び1997年7月2日に出願された米国特許出願第08/887,215号に記載されたものも含まれる。これらの文献の記載内容はすべて引照を以って繰り込みここに記載されているものとみなす。

【００５５】

そのような治療剤には、白金化合物（例えば スピロプラチン，シスプラチン，及びカルボプラチン），メトトレキセート，フルオロウラシル，アドリアマイシン，マイトマイシン，アンサミトシン，ブレオマイシン，シトシンアラビノシド，アラビノシルアデニン，メルカプトポリリジン，ビンクリスチン，ブスルファン，クロラムブチル，メルファラン（例えば ＰＡＭ，Ｌ－ＰＡＭ又はフェニルアラニンマスタード），メルカプトプリン，ミトタン，プロカルバジンヒドロクロライドダクチノマイシン （アクチノマイシンＤ），ダウノルビシンヒドロクロライド，ドキソルビシンヒドロクロライド，タキソール，プリカマイシン（ミトラマイシン），アミノグルテチミド，エストラムスチンリン酸ナトリウム，フルタマイド（flutamide），酢酸ロイプロライド（leuprolide），酢酸メゲストロール，クエン酸タモキシフェン，テストラクトン，トリロスタン，アムサクリン （ｍ－ＡＭＳＡ），アスパラギナーゼ（Ｌ－アスパラギナーゼ）エルビナ（Erwina）アスパラギナーゼ，インターフェロン α－２ａ，インターフェロン α－２ｂ，テニポサイド（teniposide） （ＶＭ－２６），硫酸ビンブラスチン （ＶＬＢ），硫酸ビンクリスチン，ブレオマ

イシン，硫酸ブレオマイシン，メトトレキセート，アドリアマイシン，アラビノシル（arabinosyl），ヒドロキシ尿素，プロカルバジン，ダカーバジン，エトポシド及び他のビンカアルカロイドのような有糸分裂阻害剤を含む抗悪性腫瘍薬；放射性ヨウ素，サマリウム，ストロンチウムコバルト，イットリウム等（但しこれらに限定されない）のような放射性医薬品；成長ホルモン，ソマトスタチン，プロラクチン，甲状腺，ステロイド，アンドロゲン，プロゲスチン，エストロゲン及び抗エストロゲンのような（但しこれらに限定されない）ホルモンを含むタンパク質及び非タンパク質性自然生産物又はそれらのアナログ／ミメティクス；オーラノフィン，メトトレキサート，アザチオプリン，サルファザラジン（sulfazalazine），レフルノマイド（leflunomide），ハイドロクロロキン，及び エタネルセプト（etanercept）のような抗リウマチ薬を含むがこれらに限定されない鎮痛薬；バクロフェン，ダントロレン，カリソプロドール，ジアゼパム，メタキサロン（metaxalone），シクロベンザプリン，クロルゾキサゾン，チザニジンのような筋弛緩剤；コデイン，フェンタニール，ヒドロモルホン，レアボルファノール（lleavorphanol），メペリジン，メタドン，モルフィン，オキシコドン，オキシモルホン，プロポキシフェンのような麻薬性作用物質；ブプレノルフィン，ブトルファノール，デゾシン（dezocine），ナルブフィン（nalbuphine），ペンタゾシンのような麻薬性作用物質－拮抗物質；ＡＳＡ，アセトミノフェン（acetominophen），トラマドール又はそれらの組み合わせを含むナルメフェン（nalmefene）及び ナロキソンその他の鎮痛薬のような麻薬性拮抗物質；セレコキシブ（celecoxib），ジクロフェナク，ジフルニサル，エトドラク（etodolac），フェノプロフェン，フルルビプロフェン，イブプロフェン，インドメタシン，ケトプロフェン，ケトロラク（ketorolac），ナプロキセン，オキサプロキセン（oxaproxen），ロフェコキシブ（rofecoxib），サリサレート（salisalate），スルジンダク（suldindac），トルメチンを含むがこれらに限定されない非ステロイド系抗炎症剤；エトミデート，フェンタニル，ケタミン，メトヘキシタル，プロポフォル（propofol），スフェンタニル，チオペンタール等のような麻酔剤及び鎮痛剤；パンクロニウム，アトラクリウム，キサトラクリウム（cisatracurium），ロクロニウム（rocuronium），スクシニルコリン，ベルクロニウム（vercuronium）のような（但しこれらに限定されない）神経筋遮断剤；アンホテリシンＢ，クロトリマゾール，フルコナゾール，フルシトシン，グリセオフルビン，イトラコナゾール，ケトコナゾール，ニスタチン及びテルビナフィンを含む抗真菌剤、アミノグリコシドを含む抗菌剤；駆虫剤；クロロキン，ドキシサイクリン，メフロキン（mefloquine），プリマキン，キニーネのような抗マラリア剤；ダプソン，エタンブトール，エチオナミド，イソニアジド，ピラチナミド，リファブチン（rifabutin），リファンピン，リファペンチン（rifapentine）を含む抗放線菌剤；アルベンダゾール，アトバコン（atovaquone），イオドキノール（iodoquinol），イベルメクチン,メベンダゾル，メトロニダゾール，ペンタミジン，プラジカンテル，ピランテル，ピリメタミン，チアベンダゾールを含む抗寄生虫剤；アバカビル（abacavir），ジダノシン，ラミブジン，スタブジン（stavudine），ザルシタビン，及びジドブジン 並びにインジナビル及び関連化合物のようなプロテアーゼ阻害剤，シドフォビル（cidofovir），フォスカーネット及びガンシクロビルを含むがこれらに限定されない抗－ＣＭＶ剤を含む抗ウイルス剤；アマタジン（amatadine），リマンタジン（rimantadine），ザナミビル（zanamivir）を含む抗ヘルペス剤；インターフェロン，リバビリン，レベトロン（rebetron）；カルバペネン（carbapenems），セファロスポリン，フルオロキノン（fluoroquinones），マクロライド，ペニシリン，スルホンアミド，テトラサイクリン，並びに、アズトレオナム，クロラムフェニエオール（chloramphenieol），ホスホマイシン，フラゾリドン，ナリジクス酸，ニトロフラントイン，バンコマイシン等を含むその他の抗生物質；硝酸塩，利尿剤，ベータ遮断薬，カルシウムチャンネル遮断薬，アンギオテンシン転換酵素抑制薬，アンギオテンシンレセプタ拮抗剤，抗アドレナリン剤，抗律動不整剤，抗高脂血症剤，血小板凝集阻害化合物，昇圧剤，血栓溶解剤，座瘡調製剤，乾癬剤を含む抗高血圧剤；コルチコステロイド；アンドロゲン，アナボリックステロイド，ビスホスホン酸塩；スルホノ尿素（sulfonoureas）及びその他の抗糖尿病剤；通風関連薬；抗ヒスタミン剤，鎮咳剤，鬱血除去剤及び去痰剤；制酸

剤，５－ＨＴレセプタ拮抗剤，Ｈ２拮抗剤，ビスマス化合物，プロトンポンプ阻害剤，下剤，オクトレオチド及びそのアナログ／ミメティクス；抗凝血剤を含む抗潰瘍剤；免疫用抗原，イムノグロビン（immunoglobins），免疫抑制剤；抗痙攣剤，５－ＨＴレセプタ作用物質，他の偏頭痛治療剤；抗コリン作用剤及びドーパミン作用剤を含む抗パーキンソン症候群剤；エストロゲン，ＧｎＲＨ作用物質，プロゲスチン，エストロゲンレセプタモジュレータ，子宮収縮抑制剤，子宮収縮剤（uterotnics），ヨウ素産物のような甲状腺剤、及び抗甲状腺剤；非経口の鉄，ヘミン，ヘマトポルフィリン及びそれらの誘導体のような血液産物、が含まれるがこれらに限定されない。

【００５６】

遺伝子材料には、例えば、核酸，組換えＲＮＡ及びＤＮＡ、及びアンチセンスＲＮＡ及びＤＮＡを含む、天然型又は合成型のＲＮＡ及びＤＮＡ；ハンマーヘッドＲＮＡ，リボザイム，マンマーヘッドリボザイム，抗原核酸（一本鎖及び二本鎖ＲＮＡ及びＤＮＡ及びそれらのアナログ），リボオリゴヌクレオチド，アンチセンスリボオリゴヌクレオチド，デオキシリボオリゴヌクレオチド，及びアンチセンスデオキシリボオリゴヌクレオチドが含まれる。使用可能な他のタイプの遺伝子材料には、例えば、プラスミド，ファージミド，コスミド，酵母人工染色体，及び 欠陥又は「ヘルパー」ウイルスのような発現ベクターにより運ばれる遺伝子，ホスホロチオネート及びホスホロジチオネートオリゴデオキシヌクレオチドのような抗原核酸（一本鎖及び二本鎖ＲＮＡ及びＤＮＡ及びそれらのアナログ）が含まれる。更に、遺伝子材料は、例えば、タンパク質又は他のポリマーと組み合わせてもよい。

【００５７】

上述のとおり、エマルジョンナノパーティクルは、天然状態又は化学的に処理された（錯体を形成した）状態のガドリニウム，マグネシウム，鉄，マンガンを含むがこれらに限定されない常磁性又は超常磁性成分をパーティクルに取り入れてもよい。同様に、天然状態又は化学的に処理された（錯体を形成した）状態のポジトロン放射体，ガンマ線放射体，ベータ線放射体，アルファ線放射体を含む放射性核種がパーティクル上又はパーティクル内に含まれていてもよい。これらの成分を加えることにより、温度増強超音波画像化法と組み合わせて、磁気共鳴映像法、陽電子放射断層撮影法及び核医学映像法のような他の臨床的画像化法を付加的に使用することが可能となる。更に、処方物内又は処方物上に取り込んだ金属イオンを、局所的高熱を増加ないし実現する「種」として利用することも可能である。

【００５８】

更に、光学的映像法は、これは、紫外線領域から赤外線領域の電磁エネルギーによって患者の組織又は部位を照射することにより生成される患者の当該組織又は部位の可視的表示と、当該照射の結果として生じる反射、散乱、吸収及び／又は蛍光エネルギーの何れかの分析に関するものであるが、温度依存性ターゲットエマルジョンの増強された音響反射率と組み合わせてもよい。例えば、光学的映像法には、可視光写真及びその変形、紫外線映像法、赤外線映像法、蛍光測定法、ホログラフィ、可視光顕微鏡検査、蛍光顕微鏡検査、分光測定法、分光法、蛍光偏光法等が含まれるがこれらに限定されない。

【００５９】

光活性剤は、診断又は治療に適用可能な、光に対し活性があるか又は光に対し反応を示す化合物又は材料であるが、これには、例えば、 発色団（例えば所定の波長の光を吸収する材料），フルオロフォア（例えば所定の波長の光を放射する材料），光増感剤（例えばin vitro及び／又はin vivoにおいて組織壊死及び／又は細胞死を可能にする材料），蛍光材料，リン光材料等が含まれる。 「光」は、紫外線波長領域、可視光波長領域及び／又は赤外線波長領域を含む全ての光源に関する。本発明で使用可能な好適な光活性剤としては、第三者によって開示されたもの（Unger et al 6,123,923）があり、これについては引照を以って繰り込みここに記載されたものとするが、これには、例えば、 フルオレセイン，インドシアニングリーン，ローダミン，トリフェニルメチン（triphenylmethines），ポリメチン（polymethines），シアニン，フラーレン，オキサテルラゾール（oxatel

lurazoles），ベルジン（verdins），ロジン，ペルフィセン（perphycenes），サプフィリン（sapphyrins），ルビリン（rubyrins），コレステリル４，４－ジフルオロ－５，７－ジメチル－４－ボラ（bora）－３ａ，４ａ－ジアザ－ｓ－インダセン（indacene）－３－ドデカノエート（dodecanoate），コレステリル１２－（Ｎ－メチル－Ｎ－（７－ニトロベンズ－２－オキサ－１，３－ジアゾール－４－イル）アミノ）ドデカネート，コレステリルシス－パリナレート（parinarate），コレステリル３－（（６－フェニル）－１，３，５－ヘキサトリエニル）フェニル－プロピオネート（proprionate），コレステリル１－ピレンブチレート（pyrenebutyrate），コレステリル－１－ピレンデカノエート（pyrenedecanoate），コレステリル１－ピレンヘキサノエート（pyrenehexanoate），２２－（Ｎ－（７－ニトロベンズ－２－オキサ－１，３－ジアゾール－４－イル）アミノ）－２３，２４－ビスノール（bisnor）－５－コレン（cholen）－３β－オール，２２－（Ｎ－（７－ニトロベンズ－２－オキサ－１，３－ジアゾール－４－イル）アミノ）－２３，２４－ビスノール－５－コレン－３β－イルシス－９－オクタデセノエート（octadecenoate），１－ピレンメチル３－（ヒドロキシ－２２，２３－ビスノール－５－コレネート（cholenate），１－ピレン－メチル３β－（シス－９－オクタデセノイロキシ（octadecenoyloxy））－２２，２３－ビスノール－５－コレネート，アクリジンオレンジ１０－ドデシルブロマイド，アクリジンオレンジ１０－ノニルブロマイド，４－（Ｎ，Ｎ－ジメチル－Ｎ－テトラデシルアンモニウム）－メチル－７－ヒドロキシクマリン）クロライド，５－ドデカノイルアミノフルオレセイン，５－ドデカノイルアミノフルオレセイン－ビス－４，５－ジメトキシ－２－ニトロベンジルエーテル，２－ドデシルレソルフィン（dodecylresorufin），フルオレセインオクタデシルエステル，４－ヘプタデシル－７－ヒドロキシクマリン，５－ヘキサデカノイルアミノエオシン，５－ヘキサデカノイルアミノフルオレセイン，５－オクタデカノイルアミノフルオレセイン，Ｎ－オクタデシル－Ｎ'－（５－（フルオレセイニル））チオ尿素，オクタデシルローダミンＢクロライド，２－（３－（ジフェニルヘキサトリエニル）－プロパノイル）－１－ヘキサデカノイル－ｓｎ－グリセロ－３－ホスホコリン，６－Ｎ－（７－ニトロベンズ－２－オキサ－１，３－ジアゾル-４－イル）アミノ）ヘキサン酸，１－ヘキサデカノイル－２－（１－ピレンデカノイル）－ｓｎ－グリセロ－３－ホスホコリン，１，１'－ジオクタデシル－３，３，３'，３'－テトラメチル－インドカルボシアニン過塩素酸塩，１２－（９－アントロイロキシ（anthroyloxy））オレイン酸，５－ブチル－４，４－ジフルオロ－４－ボラ－３ａ，４ａ－ジアザ－ｓ－インダセン（indacene）－３－ノナン酸，Ｎ－（リサミン（lissamine）（商標）ローダミンＢスルホニル）－１，２－ジヘキサデカノイル－ｓｎ－グリセロ－３－ホスホエタノールアミン，トリエチルアンモニウム塩，フェニルグリオキサル一水和物，ナフタレン－２，３－ジカルボキシアルデヒド，８－ブロモエチル－４，４－ジフルオロ－１，３，５，７－テトラメチル－４－ボラ－３ａ，４ａ－ジアザ－ｓ－インダセン（indace ne），ｏ－フタルジアルデヒド，リサミン（商標）ローダミンＢスルホニルクロリド，２'，７'－ジフルオロフルオレセイン，９－アントロニトリル（anthronitrile），１－ピレンスルホニルクロリド，４－（４－（ジヘキサデシルアミノ）－スチリル）－Ｎ－メチルピリジニウム（methylpyridinium）ヨウ化物，クロリン類（chlorines）（例えばクロリン（chlorin），クロリンｅ６，ボネリン（bonellin），モノ－Ｌ－アスパルチルクロリンｅ６，メソクロリン（mesochlorin），メソテトラフェニルイソバクテリオクロリン及びメソテトラフェニルバクテリオクロリン），ハイポクレリン（hypocrellin）Ｂ，プルプリン類（例えば、オクタエチルプルプリン，エチオプルプリン亜鉛（ＩＩ），エチオプルプリンスズ（ＩＶ）及びエチルエチオプルプリンスズ），テキサフィリン（texaphyrin）ルテチウム，フォトフリン（photofrin），メタロポルフィリン，プロトポルフィリンＩＸ，プロトポルフィリンスズ，ベンゾポルフィリン，ヘマトポルフィリン，フタロシアニン，ナフタロシアニン，メロシアニン（merocyanines），ランタニド錯体（lanthanide complexes），フタロシアニン珪素，フタロシアニン亜鉛，フタロシアニンアルミニウム，オクタブチルオキシフタロシアニンゲルマニウム，メチルフェオホルビド－α－（ヘキシル－エーテル），ポルフィセン（porphycenes），ケトクロリン（ketochlorins），

スルホン化テトラフェニルポルフィン（tetraphenylporphines），δ－アミノレブリン酸，テキサフィリン（texaphyrins）（例えば、１，２－ジニトロ－４－ヒドロキシ－５－メトキシベンゼン，１，２－ジニトロ－４－（１－ヒドロキシヘキシル）オキシ－５－メトキシベンゼン，４－（１－ヒドロキシヘキシル）オキシ－５－メトキシ－１，２－フェニレンジアミン，及びテキサフィリン金属錯体（金属Ｙ（ＩＩＩ），Ｍｎ（ＩＩ），Ｍｎ（ＩＩＩ），Ｆｅ（ＩＩ），Ｆｅ（ＩＩＩ）及びランタニド金属Ｇｄ（ＩＩＩ），Ｄｙ（ＩＩＩ），Ｅｕ（ＩＩＩ），Ｌａ（ＩＩＩ），Ｌｕ（ＩＩＩ）及び Ｔｂ（ＩＩＩ）を含む）を含む），クロロフィル，カロチノイド，フラボノイド，ビリン，フィトクロム，フィコビリン，フィコエリトリン，フィコシアニン（phycocyanines），レチノイン酸，レチノイン，レチナート，又は上記化合物の任意の組み合わせが含まれるがこれらに限定されない。

【００６０】

上記化合物がそれぞれ例えば蛍光材料及び／又は光増感剤の何れに該当するかは、当業者であれば容易に認識し又は容易に決定できる。ＬＩＳＳＡＭＩＮＥ（リサミン）は、Ｎ－エチル－Ｎ－［４－［［４－［エチル［（３－スルホフェニル）メチル］アミノ］フェニル］（４－スルホフェニル）－メチレン］－２，５－シクロヘキサジエン－１－イリデン］－３－スルホベンゼン－メタナミニウム（methanaminium）水酸化物，分子内塩（inner salt），二ナトリウム塩（disodium salt）及び／又はエチル［４［ｐ［エチル（ｍ－スルホベンジル）アミノ］－α－（ｐ－スルホフェニル）ベンジリデン］－２，５－シクロヘキサジエン－１－イリデン］（ｍ－スルホベンジル）アンモニウム水酸化物 分子内塩 二ナトリウム塩 (Molecular Probes, Inc., Eugene, Oreg.から商業的に入手可能）に対する商標である。本発明で使用するために好適な光活性剤としては、ほかにも、米国特許第4,935,498号に記載されたものがあり、その開示内容はすべて引照を以って繰り込みここに記載されたものとみなすが、例えば、４，５，９，２４－テトラエチル－１６－（１－ヒドロキシヘキシル）オキシ－１７メトキシペンタアザペンタシクロ－（２０．２．１．１^{3}，６．１^{8}，１１．０^{14}，１９）－ヘプタコサ－１，３，５，７，９，１１（２７），１２，１４，１６，１８，２０，２２（２５），２３－トリデカエンのジスプロシウム錯体及び２－シアノエチル－Ｎ，Ｎ－ジイソプロピル－６－（４，５，９，２４－テトラエチル－１７－メトキシペンタアザペンタシクロ－（２０．２．１．１^{3}，６．１^{8}，１１．０^{14}，１９）－ヘプタコサ－１，３，５，７，９，１１（２７），１２，１４，１６，１８，２０，２２（２５），２３－トリデカエン－１６－（１－オキシ）ヘキシルホスホラミダイトのジスプロシウム錯体がこれに含まれる。

【００６１】

更に、例えば、抗体、ペプチド断片、又は生理活性リガンドのミメティクスのようなある種のリガンドは、特異的エピトープに結合したとき拮抗剤又は作用剤としてその本来的な治療効果を発揮してもよい。例えば、新生血管内皮細胞のα$_{v}$β$_{3}$インテグリンに対する抗体は、固体腫瘍の成長及び転移に対する一過性阻害を示した。α$_{v}$β$_{3}$インテグリンに向けられた治療エマルジョンパーティクルの効果は、パーティクル自体によって導入・送達される治療剤の効果に加え、ターゲティングリガンドの改善された拮抗作用によって発揮されてもよい。

【００６２】

ターゲットと結合した音響画像化物質の温度の変化は、温度の上昇又は低下の何れでもありうる。音響画像化物質の温度が上昇させられる実施形態では、エネルギー源は、エネルギーを増加するために使用され、音響反射率の増加が測定される。

【００６３】

ターゲットナノエマルジョンの部位において、超音波、短波、マイクロ波、磁気照射、電磁エネルギー又はこれらの組み合わせを含む（但しこれらに限定されない）種々の手段によって局所的高熱（高体温）を引き起こしてもよい。そのようなエネルギーは、外部のシステムにより非侵襲的に、又はカテーテルシステムにより比較的侵襲的に印加してもよい。Yangら(Med Biol Eng Comput、1979年、17巻、518-24頁）は、マイクロ波（例えば２５

００ＭＨｚ）により皮膚の表面（皮下）に対する良好な加熱が得られ、９００ＭＨｚの放射線により筋系に明らかな温度の上昇が引き起こされ、２７ＭＨｚの短波により筋層（muscle layers）に広いプラトーの温度上昇が起こる、ということを示した。これらの研究者は、局所的高熱を制御するために、波の振動数、パワー、周囲条件、血管拡張、及びコアの境界条件（core boundary conditions）どのように変化させることができるかを示した。内部に配置されマイクロ波の放射により外部から加熱される加熱ターゲットとしてのフェライト含有媒体（ferrite-impregnated medium）のフェリ磁性共鳴の使用について報告した研究者もいる。この方法により、加熱の深さは共鳴を利用しない方法に比べて５０％まで増加した。

【００６４】

侵襲が僅かしかない温熱療法は、固体腫瘍を治療するため現在利用されている癌治療法であり、この方法は、リガンド－標的化エマルジョンの温度依存性の音響的コントラスト形成を向上するための局所的発熱を生成することもできる。そのような方法により、短い時間枠の間（数マイクロ秒～数分）高温を付与することができる。侵入型（interstitial）の加熱により、標的となる組織の体積は局在化され、周囲の正常な組織に対する熱の影響は最小に留められる。侵入型加熱エネルギーは、典型的には、レーザ光、マイクロ波、又は超音波によって送達される。ターゲット部位、アプリケータの配置及び当該部位への血液の灌流に基づいてエネルギー源を選択する。アプリケータの配置に基づく距離に応じたエネルギーの減少は、アプリケータに近いターゲットに対してより重要であるが、他方、減衰に基づくエネルギーの減少は、エネルギー源から離れたところではより重要である。従って、アプリケータに近いターゲットへの適用に対しては、レーザ光（これは組織内で著しく散乱されてしまう）が好適であり、深度がより大きい（より深い位置にある）構造（組織）の加熱に対しては、超音波又はマイクロ波がより好ましい（Skinnerら、Phys Med Biol、1998年、43巻、3535-47頁）。

【００６５】

加熱効果には、好ましくは０．１Ｗ／ｃｍ^{2}より大きい強度が必要である（典型的には超音波画像化分野）と考えられている。また、強度のレベルは、（例えば約２０００Ｗ／ｃｍ^{2}のような）集中された高強度の超音波の強度より小さいものが好ましい。組織自体を損傷しないままに維持する非破壊的パルス及び強度のレベルが使用されると更に好ましい。トランスデューサの性能、識別される組織の深さ、組織の減衰、ビームの散乱及びその他の物理的特徴に応じて、強度は大きいがパルス持続時間（パルス幅）は短いものがマイクロ秒からミリ秒の範囲での適用に対して有用であると考えられている。これらのパラメータは、治療への超音波の使用に関しより一般的に使用されている収束された高強度の超音波技術からそれ自体区別される。

【００６６】

本発明の方法の他の実施形態では、音響画像化物質の温度の低下、及び反射率の減少の測定が行なわれる。温度の低下は、寒冷療法で使用される低温（cryogenic）装置のようなエネルギー吸収要素によって行なうことができる。寒冷療法は、場合により冷凍手術とも呼ばれるが、プローブを接触させて極低温を作用させることにより癌細胞を死滅させるために、プローブ内の液体窒素又は液体アルゴンをエネルギーアブソーバーとして使用する技術分野では既知である。(例えば、Leeら、Urology、1990年、54巻、135-40頁参照)。本発明の方法によれば、ターゲット組織と結合した音響画像化物質の音響反射率の減少に基づいたエネルギーアブソーバーのターゲティングのための方法と、温度の現象をモニタするための方法が提供される。

【００６７】

本発明の方法の他の実施形態では、音響画像化物質の温度を変化させるエネルギー源又はエネルギーアブソーバーが存在しない場合の音響反射率に対する、音響画像化物質の温度の変化時の音響画像化物質の音響反射率の測定が行なわれる。エネルギー源又はエネルギーアブソーバーが存在しない場合の測定は、エネルギーが変化する前か後に行なうことができる。従って、温度変化の前及び温度変化直後のコントロール条件の下で音響反射率を

測定することができる。或いは、音響反射率は、エネルギー変化が達成されたとき、及び所定時間経過後エネルギー変化が消散しターゲットがエネルギー変化以前の温度に近い温度に戻ったときに測定することもできる。

【００６８】

音響反射率の差の測定は種々の方法で、例えば、反射率の差のデジタル数値表示、示差（differential）二次元又は三次元画像、色の異なる画像等として表現することができる。

【００６９】

本発明には、上記各方法を実行する装置も含まれる。本発明の装置は、温度感受性音響画像化物質が結合されるターゲットの温度の変化を測定する。本発明の装置は、音響画像化物質の温度を変化するよう構成される要素、音響エネルギーをターゲットへ送達するよう構成される超音波源、表面の音響反射率を測定するよう構成される超音波検出要素、及び温度変化がない場合のターゲットの音響反射率に対する、温度の変化時のターゲットの音響反射率を決定するコンパレータを含む。超音波送達要素及び超音波検出要素は、少なくとも１つの超音波圧電トランスデューサを有することが好ましい。温度変化要素は、超音波源、短波源、マイクロ波源、磁気照射源、電磁エネルギー源のようなエネルギー源、又は循環液体窒素又は循環液体アルゴンを含む低温要素のようなエネルギーアブソーバーから構成されうる。エネルギー変化要素は、プローブの形態をとることが好ましい。

【００７０】

本発明の装置のコンパレータ要素（比較測定器）は、エネルギー変化要素によって引き起こされる温度変化がない場合とある場合の示差測定（differential measurement）を提供する。一実施形態では、コンパレータは、エネルギー変化がない場合とある場合にそれぞれ生成される音響反射画像の減算に基づいた示差画像（differential image）を生成する画像プロセッサを有する。画像プロセッサは、減算画像（subtraction image）を生成するためのコンピュータハードウェア及びソフトウェア要素のような画像フレーム記憶要素及び／又は電子要素を含んでもよい。

【００７１】

本発明の好ましい実施形態は、以下の実施例で具体的に説明される。特許請求の範囲の射程内に含まれる他の実施形態は、本書において開示された本発明の詳細な説明又は実務を考慮することにより当業者には明らかとなるであろう。発明の詳細な説明は、実施例も同様であるが、単なる例示と考えるべきであり、本発明の保護範囲及び技術的思想は、特許請求の範囲に示されている。

【実施例１】

【００７２】

実施例１では、ペルフルオロオクタン、ペルフルオロジクロロオクタン及びペルフルオロオクチルブロマイドにおける超音波の速度の温度依存性の測定について説明する。

【００７３】

超音波の速度は、２５ＭＨｚパナメトリクス社製球面上収束トランスデューサＶ３２４(25 MHz, Panametrics V324 spherically focused transducer)を用いて決定された。測定は、湯浴中に垂直にセットされた密閉試料容器に８ｍｌのフルオロカーボンを加え、飛びとびの温度において、ペルフルオロオクタン、ペルフルオロジクロロオクタン又はペルフルオロオクチルブロマイドに対して行なった。試料容器の背面は、ステンレス鋼製の反射体から構成され、完全に閉鎖されたウェルを超えて延在し液体（水）通路測定及び試料通路測定を考慮している。ステンレス鋼反射体が音響ホログラム形成ビーム（insonifying beam）に対し垂直に配されるように試料容器を配置した。

【００７４】

トランスデューサから試料容器の前壁までの飛行時間及びトランスデューサからステンレス鋼プレートまでの飛行時間が、試料の互いに独立した（異なる）９つの位置に関して決定された。そして音速は、各温度につきそれぞれ平均して求めた。温度は、周囲の水浴を加熱しかつ試料が平衡に達するための時間（典型的には２０～２５分）を設けることにより、２５℃から４７℃まで２℃ずつ増えるように変化させた。音速は、既に開示されてい

るアルゴリズム（Kuoら、J. Acoust. Soc. Am.、1990年、88巻、1679-82頁）を用いて計算した。

【００７５】

音速測定の結果を図１にまとめた。他の液体ペルフルオロカーボンについて既に報告されているように、音速は、上記ペルフルオロカーボンの各々に対し、温度の上昇に応じて直線的な減少を示した。

【実施例２】

【００７６】

実施例２では、アビジン－ビオチンターゲティングのためのビオチン化マイクロエマルジョンの調製について説明する。

【００７７】

ビオチン化ホスファチジルエタノールアミンをペルフルオロカーボンマイクロエマルジョンの外側の脂質単分子膜に取り込むことによりビオチン化エマルジョンを調製した。調製したマイクロエマルジョンは、以下のように、水中にペルフルオロオクタン（４０％ Ｗ／Ｖ， ３Ｍ），植物油（２％ Ｗ／Ｖ），界面活性剤共混合物（２．０％， Ｗ／Ｖ）及びグリセリン（１．７％， Ｗ／Ｖ）を含む。界面活性剤共混合物は、６４モル％レクチン(Pharmacia, Inc)，３５モル％コレステロール（Sigma Chemical Co.)及び １モル％ Ｎ－（６－（ビオチノイル）アミノ）ヘキサノイル）－ジパルミトイル－Ｌ－α－ホスファチジル－エタノールアミン，Pierce, Inc.）をクロロホルム中に溶解することにより調製した。クロロホルム－脂質混合物は、減圧蒸発し、５０℃の真空窯で一晩乾燥し、そして超音波処理により水中へ分散させた。そして、懸濁液を、フルオロカーボン、植物油、グリセリン、及び脱イオン蒸留水と共に、ブレナーカップ（Dynamics Corporation of America）に移し、３０～６０秒間乳化処理した。乳化混合物をMICROFLUIDICS emulsifier (Microfluidics Co.）に移し、３分間20,000 PSIで連続的に処理した。出来上がったエマルジョンを、ガラス瓶に入れ、窒素を充填し、使用するまで圧着密閉栓（stopper crimp seal）で密閉する。パーティクルの大きさ（径）は、レーザ光散乱微小粒子径分析機（laser light scattering submicron particle size analyzer）（Malvern Zetasizer 4, Malvern Instruments Ltd, Southborough, Mass.)を用い、３７℃で３回測定した。その結果、パーティクルの大きさは、平均粒子径４００ｎｍ未満であり、径分布も狭かった。

【実施例３】

【００７８】

実施例３では、ナノパーティクルがＦ（ａｂ）フラグメントと結合するエマルジョンの調製に使用可能な方法を説明する。

【００７９】

エマルジョンのターゲティングは、脂質単分子膜に取り込まれたプライマー材料を介して、抗体をナノパーティクルに直接化学的に結合することにより達成することができる。ペルフルオロカーボンナノパーティクル造影剤（画像化剤）は、実施例１で説明したようにして調製される。

【００８０】

Ｆ（ａｂ）フラグメントは、イムノピュアＦ（ａｂ）調製キット（Pierce, Rockford, IL)を用いて生成及び単離される。簡単に説明すれば、ＩｇＧを２０ｍＭリン酸／１０ ｍＭ ＥＤＴＡバッファ（ｐＨ ７．０）で透析し、２０ｍｇ／ｍｌに濃縮し、固定化パパインで消化する。可溶化されたＦ（ａｂ）は、プロテインＡカラムを用いて、Ｆｃフラグメント及び未消化のＩｇＧタンパクから精製する。Ｆ（ａｂ）フラグメントは、Ｇ２５－１５０カラムと脱酸素リン酸バッファー（ｐＨ６．７）を用いて、過剰のシステインから精製する。フラクションの同定は、通常のごとく、ＳＤＳ－ＰＡＧＥによって行なう。

【００８１】

Ｆ（ａｂ）の各フラクションをプールし、プライマー導入エマルジョンと混合する（エマルジョン１ｍｌ当りＦ（ａｂ）１～２ｍｇ）。混合物をｐＨ６．７に調節し、窒素を封入して密閉し、外界温度で穏やかに連続的に混合して一晩反応させる。混合物は、次に、

結合しなかったＦ（ａｂ）フラグメントを除去するために、300,000 MWCO Spectra/Por DispoDialyzer (Laguna Hills, CA)を用い、１０ｍＭリン酸バッファ（ｐＨ７．２）に対して透析してもよい。最終的に得られたエマルジョンは、ガラス瓶に入れ窒素を封入し、使用するまで４℃で保存する。上記の方法において、関連性のないコントロールＩｇＧＦ（ａｂ）フラグメントを用いることにより、非特異的コントロール用エマルジョンを調製することができる。

【実施例４】

【００８２】

実施例４では、アビジン－ビオチン結合体を有するターゲットと結合したマイクロエマルジョン超音波造影剤を用いたニトロセルロース膜の温度依存性ターゲティングについて説明する。

【００８３】

音響反射後方散乱を測定するための超音波データの取得のセットアップ及び分析は以下のように行なった。２５ＭＨｚ球面状収束トランスデューサ（spherically focused transducer）（直径０．６３ｍｍ，焦点距離２．５４ｍｍ，Panametrics V324）を３つの垂直そり（orthogonal sleds）からなるガントリーに取り付けた。トランスデューサは、１００μｍの分解能を持つコンピュータ制御運動コントローラ（Aerotech Unidex 12）により、ラスタ走査パターンに従って摺動された。モータコントローラからモータに送信されるパルス列は、デジタルカウンタ（National Instruments PCI-1200）で計数され、そして、デジタル遅延生成器（digital delay generator）（Stanford Research Systems DG535）のために（１つの）トリガが生成された。そして、遅延生成器は、パルス発生器（Panametrics 5900）とデジタイジング（digitizing）オシロスコープ（Hewlett-Packard 54510B）とに対し（１つの）トリガを送信し、リアルタイムデジタイザ（Tektronix RTD720A）に対し（１つの）遅延トリガを送信した。トランジューサが６８×６８（６．８ｍｍ×６．８ｍｍ）グリッド内の凝（血）塊（clot）の表面に亘って１００μｍの分解能で走査されるとき、後方散乱された超音波を表すトレースが、実行中に捕捉された。そして、トレースは、画像再構成とデータ記憶のために、リアルタイムデジタイザからＧＰＩＢを介して制御コンピュータ（Apple Power Macintosh 7300）へ伝送された。データ取得には、典型的に、１スキャン当り約４分かかった。

【００８４】

試料容器は、試料の音響ホログラム（insonification）の形成を可能にする音響アパーチャを有する完全に閉鎖されたウェルから構成される。試料容器は、２つのポートを介し、試料への造影剤の散布を可能にするシリコンチューブ（Masterflex Platinum, 内径1/8インチ）に取り付けられた。２０ｍＬ／ｍｉｎの速度の流量を供給するためにローラポンプ（roller pump）（Masterflex, Cole-Panner Inc.）を使用した。フローシステムには、５０ｎｍのリン酸バッファを２０ｍＬ充填した。試料容器及び封入された試料は、垂直に載置され、造影剤の受身的な（passive）沈殿が起こらないようにした。試料を最初に配置した後、注入ポートを介して１００μＬの造影剤を瞬時投与し、最初と、６０分の照射後に超音波モニタリングを行なった。そして、試料容器とチューブをリン酸バッファでフラッシュした。

【００８５】

画像形成により造影剤のターゲティングが成功したことを確認した後、温度調節器（DigiSense, Cole-Parmer Inc.）によって制御される投入ヒータを用いて２７℃から４７℃まで温度を５℃ずつ上昇した。水浴全体を磁気スターラープレート上に載置して、適切に攪拌し水浴内全体の温度分布が一様になるようにした。温度の（５℃ごとの）各測定点において、ステンレス鋼プレートからの反射を観察することによりトランスデューサの焦点を求めた。そして、試料（容器）の前壁をトランスデューサの焦点の位置にセットした。

【００８６】

反射された超音波信号は全波整流され、ピーク検出ｃ－スキャン画像の描画に使用されて、凝塊又はニトロセルロース試料の周りに目的のユーザ定義領域を描くことができるよう

にした。試料の表面からの超音波の問合せ波（interrogating wave）の反射を表す信号は、矩形ウィンドウ関数（rectangular windowing function）によって分離された。ウィンドウの位置決めは、ウィンドウのエッジをニトロセルロース紙の前壁エコーと後壁エコーの間の中間に位置付ける自動アルゴリズムによって、ニトロセルロース試料の場合注意深く調節した。そして、分離した信号を高速フーリエ変換し、使用可能な帯域幅（１７～３５ＭＨｚ，１０ｄＢ下がった点のところで求めた）に亘る平均のパワーを対数で（in the logarithmic domain）計算した。そして、この「集積されたパワー（integrated power）」は、目的の領域の全ての点に対して選別され、最も明るい１００個の点を分析のために記憶した。スキャンの際の各点において求められた集積パワーもまた凝塊の超音波増強における変化の画像を描画するために使用した。周波数依存性の反射増強は、最も明るい１００個の点に対して平均が取られ、そしてコントロールスキャンに関する反射増強を減算することにより正規化（normalize）した。各試料に対しそれぞれこのプロセスを実行した。

【００８７】

in vitroでの温度依存性ターゲットエマルジョンのアビジン－ビオチンターゲティングのために、ニトロセルロース膜は以下のようにして調製した。ニトロセルロース平膜を、ジアミノヘキサンスペーサと結合するコントラストのために調製し、タンパク質結合体（複合体）のためにグルタルアルデヒドによって活性化した。ニトロセルロースディスク（直径２．４ｃｍ）を１，６ジアミノヘキサン（２．５％Ｗ／Ｖ、ｐＨ１１．９)に浸沈して６０分間一定速度で回転的に攪拌した。次に、これらの膜を、まず１Ｍ酢酸中で約１２時間、次いで超純水中で１２時間一定速度で攪拌することにより洗浄した。この洗浄の際、酢酸と超純水は、それぞれ数回交換した。そして、ジアミノアルカンで修飾されたニトロセルロース膜を１５分間０．５Ｍ $NaHCO_3$／Na_2CO_3（ｐＨ１０）中の１％グルタルアルデヒドにさらし、そして超純水中で３時間洗浄した（その際超純水は数回入れ替える）。ジアミノヘキサンで修飾されグルタルアルデヒドで活性化されたニトロセルロース膜は、使用するまで４℃で乾燥保存した。

【００８８】

０．１Ｍリン酸緩衝食塩水（ＰＢＳ）(ｐＨ７．２－７．４）に溶解したアビジン（５０μｇ）を、マイクロリットルシリンジを用いて、各ニトロセルロース膜の中心にスポット滴下して風乾し、乾燥させた。次に、膜をそれぞれ、ＰＢＳ－０．１％Ｔｗｅｅｎ２０を５分毎に３回交換することによって洗浄した。２０分間アビジンを適用した後結合されずに残っているグルタルアルデヒド活性化タンパク質結合部位をブロックするために、ＰＢＳ－０．１％Ｔｗｅｅｎ２０に懸濁した脱水粉乳を加えた。過剰のタンパク質は、等張ＰＢＳの５分間洗浄を３回繰り返して除去した。

【００８９】

５つのアビジン導入ニトロセルロースディスクと５つのコントロールニトロセルロースディスクを、ビオチン化ペルフルオロオクタンパーティクルに曝すために使用した。音響画像化により造影剤のターゲティングが成功したことを確認した後、温度調節器（DigiSense, Cole-Parmer Inc.）によって制御される投入ヒータを用いてターゲット試料の温度を２７℃から４７℃まで５℃ずつ増加した。水浴全体を磁気スターラープレート上に載置して、適切に攪拌し水浴内全体の温度分布が一様になるようにした。温度の（５℃ごとの）各測定点において、ステンレス鋼プレートからの反射を観察することによりトランスデューサの焦点を求めた。そして、試料（容器）の前壁をトランスデューサの焦点の位置にセットした。

【００９０】

図２に、ターゲット及びコントロールニトロセルロース膜に関する温度に応じた超音波反射力の変化を示した。これらの膜には、アビジンが一滴滴下されており、そのスポットはほぼ完全な円形をなしている。図２の各写真は、２７℃の基準温度から各温度の測定点のそれぞれへ移行する場合の反射の増強に関する変化を示している。色の濃い部分は、濃さが大きいほど増強の変化も大きいことを表しており、コントロールニトロセルロースと比

較すると、ターゲットニトロセルロースではかなり増加していることを見出すことができる。

【００９１】

ニトロセルロース膜試料の各々に対し最も明るい１００個の部位を選択し、平均の周波数依存性反射増強を求めた。そして、各曲線を正規化して、同じ温度のコントロールニトロセルロース膜に対する反射増強を求めた。

【００９２】

更に、温度と共に増加する超音波後方散乱増強を、帯域幅(17～34 MHz)を限定した平均によって図３に示した。結合ナノパーティクルと基質との間の温度の増加と共に生じるインピーダンスのミスマッチの増加により、ターゲット基質からの反射増強は改善される。ニトロセルロースに対するコントラストの増強と温度との間の相関（Ｒ＝０．９５）は優れている。図３に、反射増強の変化を温度の関数として定量化した。全体として、反射増強の増加は、ニトロセルロースに対し０．０８ ｄＢ／Ｃである。

【実施例５】

【００９３】

実施例５では、アビジン－ビオチン結合体を有するターゲットと結合したマイクロエマルジョン超音波造影剤を用いたニトロセルロース膜上の血漿の豊富な血漿凝血塊の温度依存性ターゲティングについて説明する。

【００９４】

ビオチン化１Ｈ１０抗体を、ＥＺ－ＬＩＮＫ Ｓｕｌｆｏ－ＮＨＳ－ＬＣ－ビオチン化キットを用いて調製した。簡単に説明すると、リン酸緩衝食塩水１ｍＬ中の抗体２～１０ｍｇを蒸留水中のＳｕｌｆｏ－ＮＨＳ－ＬＣ－ビオチン混合して、１２～２０倍Ｍの過剰試薬を抗体に供した。そして、この溶液を室温で３０分間インキュベートした。ビオチン化された抗体は、１０ｍＬ脱塩カラムを用いて試薬から分離した。リン酸緩衝食塩水で平衡と溶出を行なった。溶出液のフラクションを収集し、ＵＶ吸光度を分光光度計を用いて２８０ｎｍで測定した。抗体を含むフラクションは、使用するまで４℃で保存した。

【００９５】

膜上に置いたプラスチックモールド内で、ヒトクエン酸添加血漿（３７５μｌ）及び１００ｍＭ塩化カルシウム（２５μｌ）を３単位のトロンビンと混合することにより、ニトロセルロース膜表面上に血漿凝血塊が生成した。血漿は外界温度でゆっくりと凝固させ、そしてＰＢＳに移してコントロール又はターゲットコントラストシステムに曝すまで保存した。

【００９６】

凝血塊（ｎ＝９）をビオチン化１Ｈ１０抗体と共にＰＢＳ中で穏やかに攪拌しながら４℃の冷蔵室内で一晩インキュベートした。そして、凝血塊をリンスし、室温で１時間穏やかに攪拌しながら１００μｇのアビジンに曝した。そして、凝血塊を再びリンスし、実施例４に記載したように造影剤に曝す準備をした。ビオチン化抗体又はアビジンでプレターゲットしていないコントロール凝血塊（ｎ＝７）を同じ方法でビオチン化造影剤に曝した。

【００９７】

実施例４と同様に音響反射率の測定を行なった。自然のヒト血漿凝血塊の本来的に乏しいエコー発生性を図４の左側の部分に示した。図４に、造影剤導入前後の血漿凝血塊の集積反射力に関する２つの画像を示した。これらの画像のグレースケールは、反射増強の対数で表されている。右側の写真の色のより濃い部分は、部位特異的超音波造影剤の存在によって凝血塊の増強が増加されている領域を表している。

【００９８】

図４に示したものと同じ血漿凝血塊を図５にも示したが、図５では、グレースケールは、２７℃の基準温度から５℃ずつの温度の上昇により引き起こされた増強の変化を表している。ターゲットニトロセルロースの場合と同様に、ターゲットヒト血漿凝血塊も、温度に従いエコー発生性にかなりの増加を示し、他方コントロール凝血塊（これらは造影剤に曝されていない）は、この温度の範囲では殆ど変化を示さない。図６に、トランスデューサ

の使用可能な帯域幅（１７～３４ＭＨｚ）に亘って平均された凝血塊の最も明るい１００個の部位に関する反射増強の結果を示した。この反射増強対温度のプロットに直線を嵌め込むと相関係数Ｒ＝０．９９と傾き０．２１ｄＢ／Ｃが得られる。

【００９９】

比較を行なうと、コントロールニトロセルロースとコントロールヒト血漿凝血塊は、何れも、温度の上昇に対し反射力の僅かな変化しか示さなかった。これに対し、ターゲット又は増強基質は、何れも、５℃程度の温度の変化に対しても（反射）増強に関し検出可能な変化を示した。この示差音響反射応答（differential acoustic reflectivity response）は、コントラストのあるターゲット及び非ターゲット表面の間のセグメント形成、及びカラー化、デジタル減算（subtraction）又はその他の類似の方法による画像表現の向上に利用することができる。ターゲット組織の検出感度の向上、並びに臨床上の読取者への表示及び該読取者の認識を改善する能力により、その他の方法では正しく認識できなかったような病状の認識を大きく改善することができるであろう。

【実施例６】

【０１００】

実施例６では、ヒト血漿凝血塊と結合したアビジン－ビオチンターゲットフルオロカーボンエマルジョンの音響反射率に関する温度依存性の変化のヒステリシスの欠如について説明する。

【０１０１】

温度の効果の可逆性を試験するために、２つのヒト血漿凝血塊試料を加熱してから冷却し、実施例３に記載した方法を用いて温度の各測定点に対して反射（率）増強を記録した。ヒステリシス曲線を図７にプロットした。測定の誤差バーの範囲内では、増強の大きさは、各温度において同一であり、温度の変化の方向に依存していなかった。選択されたフルオロカーボン、即ちペルフルオロオクタンの沸点（１０５℃）及び試料がその中で音響ホログラム化される３７℃の水浴の熱消散効果を考慮すれば、この結果により、ターゲット組織の音響反射の増強又は減少は、結合エマルジョンパーティクルの音響インピーダンスの温度依存性の変化に基づくのであって、フルオロカーボンの気体状態への相転移に基づくのではないということが確認される。

【実施例７】

【０１０２】

実施例７では、特に腫瘍量の形態の検出、局在化及び画定（defining）に関する高熱（高体温）療法と組み合わせた本発明の超音波画像化への適用及び高熱のモニタリングに使用可能な方法及び装置について説明する。

【０１０３】

現在、種々の癌、とりわけ８ｃｍ未満の深さの癌に対する効果増強（augmentive）治療としての放射線腫瘍学と組み合わせた種々の形態の温熱療法が使用されている。この応用に対して現在使用されている装置の１つとして、Labthermics Technologies, Champaign, IL製のSonotherm（登録商標）1000がある。適切な組織温熱療法は、周囲の正常組織に対する処置を最小にして腫瘍を加熱することに関わっている。SONOTHERM 1000は、特定の組織量を小さな立方体の治療ボクセルに分ける能力を有する。超音波アレイの異なる成分の周波数と強度を調節することにより、「適切な」加熱パターンが達成される。

【０１０４】

とりわけ小さい腫瘍を識別することの困難性に関する、この療法全体に関する鍵は、（１）三次元体積空間における腫瘍の正確な局在化及び形態描画、及び（２）腫瘍死滅と正常側枝組織の保存を保証する組織加熱プロセスにおける非侵襲型温度測定である。本発明の温度依存性超音波造影剤は、とりわけ癌が小さいか、バックグランドが本来的に音響反射性である場合に、高分解能検出、局在化、二次元又は三次元空間における腫瘍のマッピングを大きく増強する。このことは、ナノパーティクルターゲット組織と周囲の正常組織の示差（differential）超音波応答によって達成される。更に、音響後方散乱の温度依存性の変化は内部温度測定として使用することもできるため、ターゲット組織を適切なレベル

に加熱し、他方他の組織の加熱を最小化することを保証する。この高分解能非侵襲型温度測定は、操作者が組織の温度をモニターしかつ温熱療法プロトコールを手動で調節できるようにするために、スケール化（scaled）カラーマップを使用することにより常にリアルタイムで表示することもできる。或いは、自己モニタリングアルゴリズムを有する温熱装置により、「実行中に」自動で超音波ビームを調節することもできる。

【０１０５】

腫瘍のような病状は、通常の超音波によっては正常な組織から区別することが困難であり、特殊なコントラスト増強から利益が得られることがしばしばある。不利なことには、隣接する組織の大きな音響反射率は、ターゲット分子造影剤を用いた場合であっても、達成されるコントラストの増強の大きさを減じることがよくある。温度依存性ターゲット分子造影剤を使用する可能性により、更に、正常周囲組織に対するターゲット組織からの音響信号を増加することによって病的組織から正常組織を区別するための独特の機構が提供される。この特徴により、生検又は外部放射又は温熱療法のための、腫瘍、とりわけ小さい腫瘍の区分（セグメンテーション）及び局在化が改善される。改善された局在化により、外部の治療エネルギー源のより正確なフォーカシングが可能となり、治療の効率は最大化されかつ側枝（二次的）損傷は最小化される。更に、周囲組織に対し増加された音響後方散乱の大きさにより、非侵襲型の温度測定が提供される。ターゲット部位における適切なレベルの加熱をモニターし及び手動で制御するか高熱装置内部の自己制御システムによって（自動的に）制御することもできる。技術上の利点から、直ちに乳癌、悪性黒色腫、肉腫、リンパ腫、頭部及び頸部癌の治療、及び近いうちに、大腸（結腸）、子宮頸、子宮、肝臓、膵臓、胃等のようなより深い位置に生じる腫瘍の治療に対する適用を考えることができる。

【０１０６】

例えば、乳癌の患者又は動物が病院に入院し、静脈カテーテルを受けるとする。新生血管細胞の$\alpha_v\beta_3$インテグリンに向けられたモノクローナル抗体フラグメントを有する温度感受性音響ナノパーティクルを、０．１～１．０ｍＬ／ｋｇ（体重）、好ましくは０．２５～０．５ｍＬ／ｋｇ（体重）の量で投与する。剤を１５分～５時間、好ましくは１～２時間循環させて、新生血管組織レセプタを飽和させる。Agilent (Andover, Mass.), Acuson (Mountainview, Calif.), ATL (Bothel, Wash.), GE (Farifield, Conn.) Toshiba (Tokyo, JP)製の装置及び類似の装置のような商業的に入手可能な標準的な超音波画像形成装置を使用することにより基準の超音波画像が得られる。音響温度記録法（Sonothermography）は、SONOTHERM 1000 Therapy system (Labthermics Technologies, Champaign, Ill.) 又は製造業者の推薦する関連装置によって、疑わしい塊の存在が予想される位置に対して行なうことができる。SONOTHERM 1000は、選択された領域の組織の温度（４２℃～４５℃）に間欠的上昇（持続時間はそれぞれ１０秒未満）を繰り返し引き起こす。ナノパーティクルで標的された腫瘍血管系と周囲の正常組織の音響コントラストの示差的変化（differential changes）が、腫瘍量（tumor burden）のモルフォロジを特異的に検出、局在化及び画定するために使用される。これらの結果は、SONOTHERM 1000のプログラムに組み込まれ、与えられるべき高熱照射のための位置及び分布はより正確にされる。続いて行なわれる高温療法セッション中はいつでも、ターゲット組織の各領域内におけるターゲットナノパーティクルにより与えられる音響後方散乱の温度依存性の変化を、深部の腫瘍温度を非侵襲的に決定するために使用することもできる。この情報により、できるだけ厳しい許容度で高温プロトコールを精密かつ連続的に制御することができるため、正常組織の側枝（二次的）損傷は最小化され、この方法の全体としての安全性も向上する。

【実施例８】

【０１０７】

実施例８では、カテーテル用侵襲型温熱療法に本発明を適用するのに使用可能な方法及び装置について説明する。

【０１０８】

標的化された温度依存性ナノパーティクルは、以下のようにカテーテルシステムで使用す

ることもできる。治療及び手術に適用するために高度に収束された熱を生成することが可能な種々の治療用超音波カテーテルが開発された。そのような装置の一例として、Leeら(IEEE transactions of Biomedical Engineering. 1999年、4巻、880-90頁）によって記載されたマルチエレメントアレイシステム（multielement array system）がある。このユニットは、in vitro及びin vivoの両方で実施され、３０ｍｍ×３０ｍｍ×３５ｍｍのターゲット体積の９２％以上で治療温度の上昇（５℃以上）を達成した。これ及び類似の装置により、温度分布の精密な制御を行なうことができる。この温熱（療法）カテーテルシステムを、商業的に利用可能な静脈内超音波トランスデューサ技術と組み合わせることにより、１０～５０ＭＨｚの範囲の周波数における加熱された組織の精細かつ緻密な超音波画像を（両者に対し）生成することができた。これらの映像化／治療カテーテルデュアルシステムは、内視鏡及び静脈内適用の両方に使用することができた。

【０１０９】

温熱療法及び超音波システムのためのカテーテルは、以下のような癌の治療に使用することができる。膵臓癌が疑われる患者を病院に入院させ、静脈カテーテルを行なう。新生血管細胞の$\alpha_v\beta_3$インテグリンに向けられたモノクローナル抗体フラグメントを有する温度感受性音響ナノパーティクルを、０．１～１．０ｍＬ／ｋｇ（体重）、好ましくは０．２５～０．５ｍＬ／ｋｇ（体重）の量で投与する。剤を１５分～５時間、好ましくは１～２時間循環させて、新生血管組織レセプタを飽和させる。治療／画像化超音波組合せカテーテルを進行させ、経胃／経十二指腸（transgastric/transduodenal）アプローチからの膵臓の画像を得る。膵臓腫瘍の診断、位置及び程度が上述のような温度依存性画像化プロトコールによって確認される。局所的温熱療法を行なうために腫瘍の音響ホログラムを生成する。ターゲット組織の温度は、音響後方散乱の変化によって連続的にモニターされる。漸増する温度変化は、繰り返し更新され操作者によって観察される超音波画像ディスプレイ上でカラーマッピングされる。腫瘍破壊を最適化し、正常組織の二次的（側枝）損傷を最小化するために、操作者が手動で又は装置が自動的に超音波ビームの強度又は周波数を調節する。

【０１１０】

上述したことを鑑みると、本発明の幾つもの利点が達成され、他の有利な結果が得られる事が分かる。

【０１１１】

本発明の範囲から逸脱しない範囲において上記の方法及び構成に種々の変更を行なうことができるが、そのため上記の説明に含まれる全ての内容は単なる例として説明したのであって、これらに限定することは意図しない。

【０１１２】

この明細書で引用した文献はすべて、引照によって本書に繰り込まれているものとする。これら文献に関する議論は、その著者によりなされた主張を単にまとめたものに過ぎず、何れの文献も従来技術を構成するものとは認めない。出願人は、上記各文献の正確さ及び適切さについて争う権利を留保する。

【図面の簡単な説明】

【０１１３】

【図１】媒質ペルフルオロカーボン、即ちペルフルオロオクタン（ＰＦＯ）、ペルフルオロジクロロオクタン（ＰＦＤＣＯ）、及びペルフルオロオクチルブロマイド（ＰＦＯＢ）に関する、温度上昇に伴う音の伝播速度の減少。

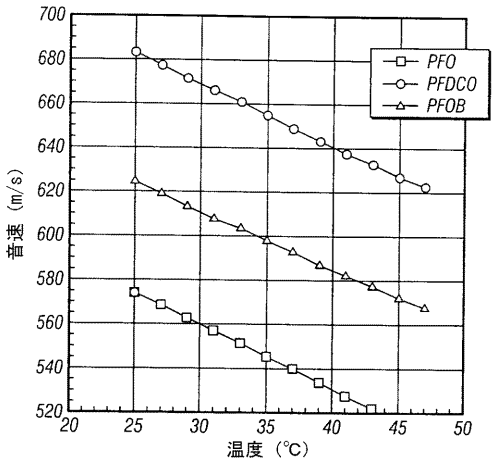
【図２】温度３２℃、３７℃、４２℃、及び４７℃に関し、２７℃で測定された反射率を超える反射率の増加を示すニトロセルロース膜から得た示差（differential）超音波画像。各図において、灰色が濃いほど反射率の増加はより大きい。

【図３】ターゲットニトロセルロース膜とコントロールとの間の、温度の関数としての超音波反射力の相対変化。

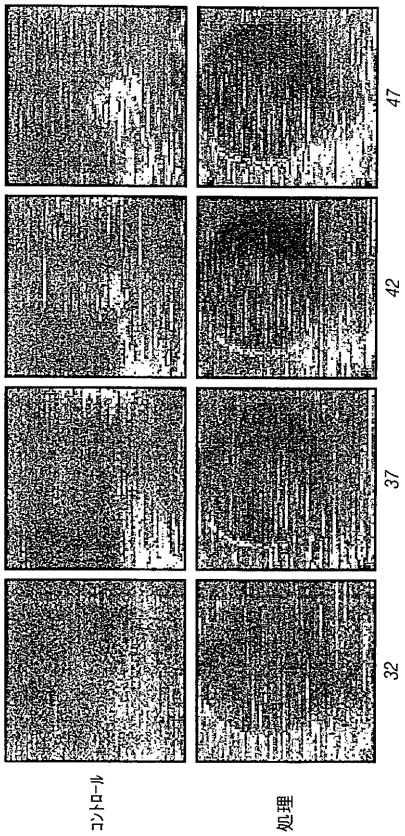
【図４】造影剤によるターゲティング前後（前：左図、後：右図）におけるヒトフィブリン凝（血）塊からの超音波反射。各図において、灰色が濃いほど反射も大きい。

【図５】温度３２℃、３７℃、４２℃、及び４７℃に関し、２７℃で測定された反射率を超える反射率の増加を示すニトロセルロース膜上のヒト血漿凝血塊から得た示差超音波画像。各図において、灰色が濃いほど反射率の増加はより大きい。
【図６】ニトロセルロース膜上のターゲットヒト血漿凝血塊とコントロールとの間の、温度の関数としての超音波反射力の相対変化。
【図７】加熱（■印）後冷却（○印）した場合の、温度の関数としてプロットされたヒト血漿凝血塊からの反射増強のヒステリシスの欠如。

【図１】



【図２】



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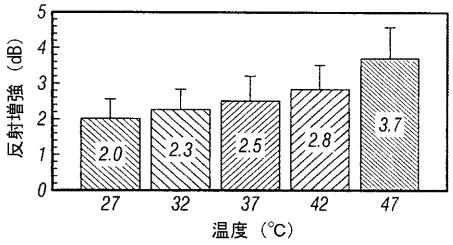
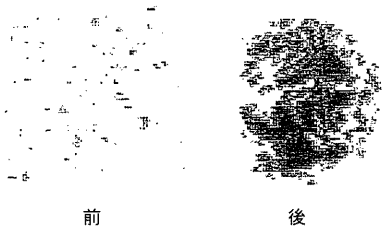
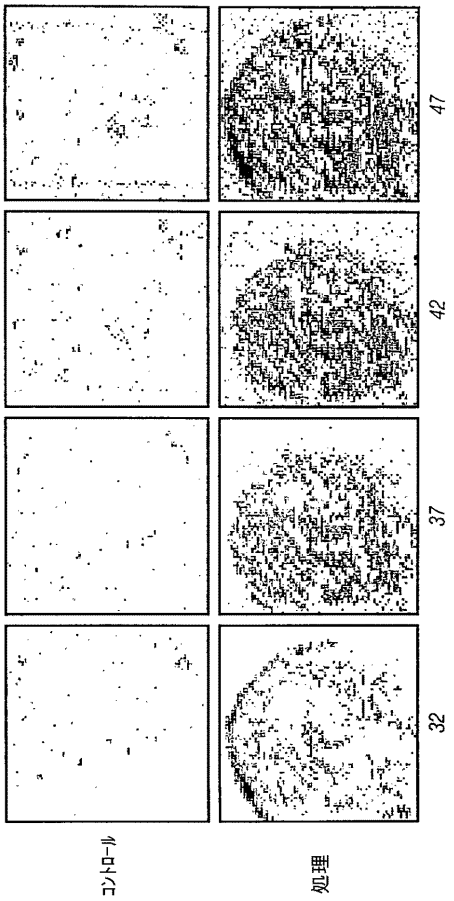

反射増強（dB）
0
1
2
3
4
5
2.0
2.3
2.5
2.8
3.7
27
32
37
42
47
温度（℃）

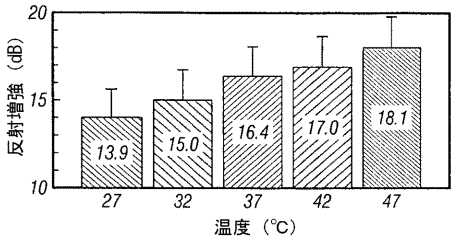
【図４】


前
後

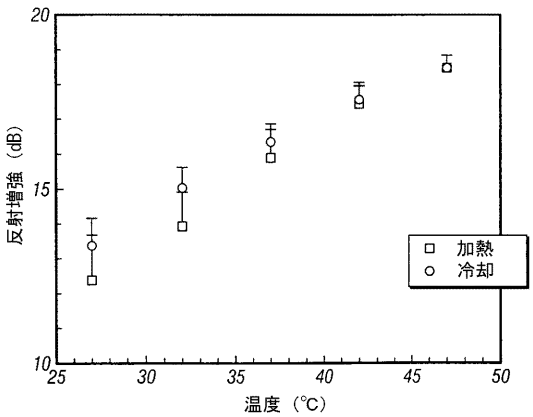
【図５】


47
42
37
32
コントロール
処理

【図６】


反射増強（dB）
10
15
20
13.9
15.0
16.4
17.0
18.1
27
32
37
42
47
温度（℃）

【図７】


反射増強（dB）
10
15
20
25
30
35
40
45
50
加熱
冷却
温度（℃）

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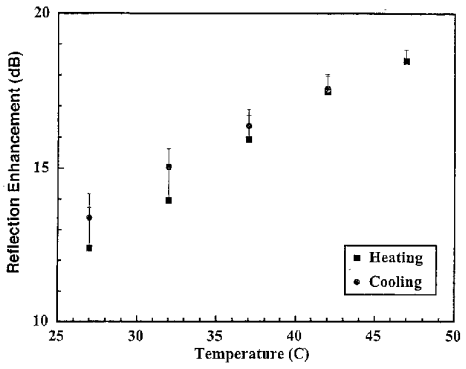

(57) Abstract: Methods and devices for enhanced ultrasound detection based upon changing temperature and ultrasound reflectivity of a temperature-dependent contrast agent bound to an ultrasound target are disclosed. The methods and devices can be used for enhanced imaging alone or in conjunction with drug delivery, with therapeutic approaches such as hyperthermia or cryotherapy or with other imaging modalities.

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ENHANCED ULTRASOUND DETECTION WITH TEMPERATURE-DEPENDENT CONTRAST AGENTS

BACKGROUND OF THE INVENTION

(1) Field Of The Invention

This invention relates generally to ultrasound detection and imaging and, more particularly, to novel compositions, methods and devices for detecting a change in ultrasound reflectivity based upon changing the temperature of a temperature-dependent contrast agent bound to the target.

(2) Description Of The Related Art

Molecular imaging can enhance the utility of traditional clinical imaging by allowing specific detection of molecular markers in tissues using site-targeted contrast agents (Weissleder, *Radiology 212*:609-614, 1999). Three approaches to site-targeted ultrasonic agents have been reported and these are based upon the use of liposomes (Alkan-Onyuksel et al., *J. Pharm. Sci 85*:486-490, 1996; Demos et al., *J. Pharm. Sci. 86*:167-171, 1997; Demos et al., *J. Am. Col. Cardiol. 33*:867-875, 1999), the use of microbubbles (Mattrey et al, *Am. J. Cardiol. 54*:206-210, 1984; Unger et al., *Am. J. Cardiol. 81*:58G-61G, 1998; Villanueva et al, *Circulation 98*:1-5, 1998; Klibanov et al, *Acad. Radiol. 5*S243-S246, 1998) or the use of nano-emulsions (Lanza et al, *Circulation 94*:3334-3340, 1996; Lanza et al, *J. Acoust. Soc. Am. 104*:3665-3672, 1998; Lanza et al, *Ultrasound Med. Biol. 23*: 863-870, 1997). Liposomes are spherical bimembrane vesicles produced spontaneously by phospholipids in water. Multilamellar lipid bilayers produced through a dehydration-rehydration process can form internal vesicles within a liposome and lead to increased acoustic reflectance (Alkan-Onyuksel et al., 1996 *supra*; Demos et al., 1997, *supra*; Demos et al., 1999, *supra*). In the second approach, microbubbles have been proposed for site-targeted modalities in addition to their perfusion applications. Microbubbles have been targeted towards thrombi (Unger et al., 1998m supra; Lanza et al., *Ultrasound. Med. Biol. 23*: 863-870, 1997), avidin-coated petri dish (Klibanov et al, 1998, *supra*) and activated endothelial cells (Villanueva et al, 1998, *supra*). Other investigators have examined the interaction of thrombus with site targeted agents. In particular, Unger et

al. has observed successful binding of MRX-408, a bubble-based contrast agent, both *in vitro* and *in vivo* (Unger et al., 1998, *supra*).

The site-targeted nano-emulsions are nongaseous acoustic contrast agents made up of lipid-encapsulated liquid perfluorocarbon nanoparticules (see Lanza et al., U.S. Patent Nos. 5,690,907,; 5,780,010; and 5,958,371). The nanoparticles are approximately 250 nm in diameter. Perfluorocarbon nanopatriculate emulsions have been shown to provide substantial acoustic contrast when targeted towards *in vitro* and *in vivo* thrombi preparations (Lanza et al., 1998, *supra*; Lanza et al., 1997, *supra*).

One of the challenges confronting the use of site-targeted contrast agents is the sensitive detection and differentiation of the particles from the surrounding soft tissue. Detection of pathological changes on or near vascular surfaces may be compromised because the targeted substrate itself is echogenic or the signal from that surface may be somewhat view or angle dependent. Imaging techniques have been developed in attempts to solve this issue. Second harmonic or harmonic and power harmonic Doppler imaging has been used to allow differentiation of microbubbles in circulation from tissue (see Burns et al. *Clinical Radiol. 51*:50-55, 1996; Kasprzak et al, *Am. J. Cardiol. 83*:211-217, 1999; Senior et al, *Am. Heart J. 139*:245-251, 2000; Spencer et al, *J. Am. Soc. Echo. 13*:131-138, 2000). However, soft tissue also exhibits a second harmonic backscattered signal. Furthermore, the contrast agent may manifest velocities too slow for the sensitivity of Doppler techniques. Unlike the resonance phenomenon responsible for enhanced backscatter cross section in microbubbles, the mechanism for increased reflection enhancement from the site-targeted nanoparticle emulsions has been reported to be due to acoustic impedance mismatch at the surface where the particles bind (Lanza et al., 1998, *supra*). Thus, although site-targeted acoustic contrast agents and, in particular, the nano-emulsion contrast agents have been used as contrast agents, the development of approaches that produce a greater degree of contrast could potentially provide further sensitivity for ultrasound molecular imaging systems.

Perfluorocarbon liquids are known to transmit ultrasound at low velocities (Lagemann et al. *J. Am. Chem Soc. 70*: 2994-2996, 1948; Gupta, *Acustica 42*:273-277, 1979). The low ultrasound velocities through these substances have been shown to be

temperature dependent in that the ultrasound velocity is decreased in a linear manner with increasing temperature (Narayana et al., *Acoustics Letters 9*:137-143, 1986). This observation was reported to be potentially applicable to the development of acoustic lenses (*Id.*). Nevertheless, the temperature-dependence of ultrasound velocity in perfluorocarbon liquids has not, heretofore, been suggested to have any applicability in ultrasound imaging systems.

Ultrasound energy has been applied in site-targeted contrast agents in ultrasound imaging methods as noted above. Much of this earlier work was directed to molecular imaging so that only low level ultrasound energy was used and no change in temperature of the targeted surface was reported to occur. In microbubble ultrasound imaging systems, sufficient energy has been applied to a liquid precursor substance to form gaseous microbubbles. (Lohrmann et al., U.S. Patent No. 5,536,489; Unger, U.S. Patent No. 5,542,935). One suggested approach has been to apply the energy to produce the phase shift *in vivo*. In such approaches, temperature changes would serve to convert the gaseous precursor to the gaseous microbubbles and none of these earlier studies disclosed or suggested changing temperature of an ultrasound contrast agent which remains in the liquid state or using the change in temperature of a nongaseous contrast agent as a basis for enhancing ultrasound detection.

Thus, there remains a continuing need for developing approaches that produce an enhanced degree of contrast and provide further sensitivity for ultrasound molecular imaging systems.

BRIEF SUMMARY OF THE INVENTION

Accordingly, the inventors herein have succeeded in discovering that changing the temperature of nanoparticles which contain a nongaseous fluorocarbon liquid and which are bound to a target, produces a detectable change in acoustic reflectivity of the target. Non-targeted regions which are adjacent to the target, but are not bound by the nanoparticles, show little or no detectable change in acoustic reflectivity. As a result, the temperature-dependent change in acoustic reflectivity of site-targeted

nanoparticles provides a sensitive measurement of ultrasound reflectivity and provides enhanced contrast imaging.

Thus, in one embodiment, the present invention is directed to a method for changing acoustic reflectivity of an ultrasound target. The method comprises (1) administering to the target, a nongaseous acoustic imaging substance which binds to the target and produces a change in acoustic reflectivity with a change in temperature and (2) changing the temperature to produce a measurable change in acoustic reflectivity of the nongaseous acoustic imaging substance bound to the target. The nongaseous acoustic imaging substance, preferably, comprises a nanoparticle emulsion which contains a liquid fluorocarbon. The nongaseous acoustic imaging substance, preferably, comprises a ligand which binds to the target.

In another embodiment, the present invention comprises a method for measuring enhanced acoustic reflectivity of an ultrasound target. The method comprises (1) administering to the target, a nongaseous acoustic imaging substance which binds to the target and produces a change in acoustic reflectivity with a change in temperature and (2) changing the temperature to produce a measurable change in acoustic reflectivity of the nongaseous acoustic imaging substance bound to the target, and (3) detecting change in acoustic reflectivity of the bound substance. Detecting the change in acoustic reflectivity, preferably, comprises (a) measuring reflectivity prior to changing the temperature of the bound substance; (b) measuring reflectivity after changing the temperature of the bound substance; and (c) determining the change in reflectivity after changing the temperature of the bound substance compared to reflectivity prior to changing the temperature of the bound substance.

Another embodiment of the present invention involves a method for monitoring temperature of a tissue in a patient. The method comprises (1) administering to the patient, a nongaseous acoustic imaging substance which binds to the tissue and changes acoustic reflectivity with changes in temperature, (2) detecting acoustic reflectivity of the nongaseous acoustic imaging substance bound to the tissue (3) calculating temperature of the nongaseous acoustic imaging substance bound to the tissue. Preferably, the method monitors a change in temperature and the method further comprises changing the temperature of the tissue and the nongaseous acoustic

imaging substance bound to the tissue. Detecting acoustic reflectivity comprises detecting the change in acoustic reflectivity of the nongaseous acoustic imaging substance bound to the tissue.

In one aspect of the present invention, the change in temperature can be produced by energizing the bound nongaseous nanoparticles to increase temperature of the bound substance and enhance acoustic reflectivity of the target. The nanoparticles can be energized by ultrasound, shortwave, microwave, magnetic radiation, electromagnetic energy or a combination thereof.

In another aspect of the present invention, the temperature of the bound nanoparticles can be decreased to produce a measurable decrease in acoustic reflectivity of the target.

The methods of the present invention can be used in conjunction with administration with a biologically active agent which is incorporated into the nanoparticle. In addition, other imaging techniques can be used with the acoustic imaging upon incorporating into the nanoparticle one or more imaging agents suitable for use in such other imaging techniques such as, for example, magnetic resonance imaging, electron spin resonance imaging, spectroscopic imaging, positron emission tomography imaging, optical imaging, x-ray imaging, nuclear medicine imaging or a combination thereof.

In another embodiment, the present invention comprises a device for measuring changes in temperature of a target to which a temperature-sensitive acoustic imaging substance is bound. The device comprises a component configured to change the temperature of the acoustic imaging substance, an ultrasound source configured to transmit acoustic energy to the target, an ultrasound detecting component configured to measure acoustic reflectivity of the surface and a comparator which determines acoustic reflectivity of the target upon changing temperature relative to acoustic reflectivity of the target in absence of changing temperature. In one aspect, the comparator determines difference in acoustic reflectivity of the target prior to and after changing temperature of the acoustic imaging substance bound to the target. In another aspect, the comparator determines the difference in acoustic reflectivity of the target upon changing temperature of the acoustic imaging substance

bound to the target, compared to acoustic reflectivity of the target after the changed temperature of the acoustic imaging substance bound to the target is diminished. The temperature changing component can comprise an energy source which increases the temperature of acoustic imaging substance bound to the target or an energy absorbing component which decreases the temperature of the acoustic imaging substance bound to the target.

The device can also comprise a component which performs at least one other imaging technique such as, for example, magnetic resonance imaging, electron spin resonance imaging , spectroscopic imaging, positron emission tomography imaging, optical imaging, x-ray imaging nuclear medicine imaging or a combination thereof.

Among the several advantages achieved by the present invention, therefore, may be noted the provision of methods for enhancing acoustic reflectivity of a target; the provision of methods for distinguishing a target tissue from surrounding tissue which is acoustically reflective; the provision of methods for detecting and monitoring temperature of a tissue; the provision of methods for detecting temperature changes in a tissue such as during a therapeutic treatment involving a change in temperature; and the provision of devices for performing such methods.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the decrease in the speed of sound transmission with increasing temperatures for the perfluorocarbons, perfluorooctane (PFO), perfluorodichlorooctane (PFDCO) and perfluorooctylbromide (PFOB).

Figure 2 illustrates the differential ultrasound images obtained from nitrocellulose membranes showing the increase in reflectivity over that measured at 27° C for temperatures of 32° C, 37° C, 42° C, and 47° C in which darker grays indicate greater enhancement of reflectivity.

Figure 3 illustrates the relative change of ultrasonic reflected power as a function of temperature between targeted and control nitrocellulose membranes.

Figure 4 illustrates the ultrasonic reflection from a human fibrin clot before (left panel) and after (right panel) targeting with contrast agent wherein darker grays represent larger reflection..

Figure 5 illustrates the differential ultrasound images obtained from human plasma clots on nitrocellulose membranes showing the increase in reflectivity over that measured at 27° C for temperatures of 32° C, 37° C, 42° C, and 47° C in which darker grays indicate greater enhancement of reflectivity.

Figure 6 illustrates the relative change of ultrasonic reflected power as a function of temperature between targeted and control human plasma clots on nitrocellulose membranes.

Figure 7 illustrates the lack of hystersis of the reflection enhancement from human plasma clot plotted as a function of temperature when heated (circles) and then cooled (squares).

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, it has been discovered that the detection of acoustic reflectivity of an ultrasound target can be enhanced by changing the temperature of a nongaseous acoustic imaging substance which is bound to the target and which exhibits a measurable change in acoustic reflectivity with a change in temperature.

The acoustic imaging substance is, preferably, a nanoparticle emulsion as has been described earlier (see U.S. Patent Nos. 5,780,010, 5,958,371 and 5,989,520). The nanoparticle emulsions of the present invention are comprised of at least two immiscible liquids which are intimately dispersed, preferably, a hydrophobic material such as an oil, dispersed in water. The emulsions are in the form of droplets or nanoparticles having a diameter which typically exceeds 0.1 μ. Additives such as surface-active agents or finely-divided solids can be incorporated into the emulsion nanoparticles to increase their stability.

The oil phase of the oil-in-water emulsion comprises, preferably, 5 to 50% and, more preferably 20 to 40% by weight of the emulsion. The oil or hydrophobic

constituent exhibits an acoustic impedance that varies with changes (i.e. positively or negatively) in temperature, preferably, a fluorochemical liquid. These include straight, branched chain and cyclic perfluorocarbons, straight , branched chain and cyclic perfluoro tertiary amines, straight , branched chain and cyclic perfluoro ethers and thioethers, chlorofluorocarbons and polymeric perfluoro ethers and the like. Although up to 50% hydrogen-substituted compounds can be used, perhalo compounds are preferred. Most preferred are perfluorinated compounds. Any fluorochemical liquid, i.e. a substance which is a liquid at or above body temperature (e.g. 37°C) at atmospheric pressure, can be used to prepare a fluorochemical emulsion of the present invention. However, for many purposes emulsions fluorochemicals with longer extended stability are preferred. In order to obtain such emulsions, fluorochemical liquids with boiling points above 50°C are preferred, and most preferred are fluorochemical liquids with boiling points above about 80°C. The guiding determinant should be that the oil, e.g. a fluorochemical, should be expected to remain in a liquid phase (less than10% gas conversion) under the intended conditions of themal induction and imaging.

Reference to the term "nongaseous" or "liquid" in the context of the nanoparticle emulsions of the present invention is intended to mean that less than 10% of the interior volume of the nanoparticles is in a gas phase per total volume of the nanoparticles (i.e. v/v), more preferably, no more than about 8% (v/v), more preferably no more than about 5% (v/v), and most preferably, no more than than 2% (v/v) or less. The term "about" as used herein is intended to encompass a range of values 10% above and below a stated value such that, for example, about 8% is intended to encompass the range of values from 7.2% to 8.8%.

The nanoparticle emulsions of the present invention are, preferably, lipid encapsulated. In a specific example, the lipid encapsulated particles may be constituted by a perfluorocarbon emulsion, the emulsion particles having an outer coating of a derivatized natural or synthetic phospholipid, a fatty acid, cholesterol, lipid, sphingomyelin, tocopherol, glucolipid, sterylamine, cardiolipin, a lipid with ether or ester linked fatty acids or a polymerized lipid.

Fluorocarbon emulsions and, in particular, perfluorocarbon emulsions are well suited for biomedical applications and for use in the practice of the present invention. The perfluorocarbon emulsions are known to be stable, biologically inert and readily metabolized, primarily by trans-pulmonic alveolae evaporation. Further, their small particle size easily accommodates transpulmonic passage and their circulatory half-life (4-8 hours) advantageously exceeds that of other agents. Also, perfluorocarbons have been used to date in a wide variety of biomedical applications, including use as artificial blood substitutes. For use in the present invention, various fluorocarbon emulsions may be employed including those in which the fluorocarbon is a fluorocarbon-hydrocarbon, a perfluoroalkylated ether, polyether or crown ether. Useful perfluorocarbon emulsions are disclosed in U.S. Pat. Nos. 4,927,623, 5,077,036, 5,114,703, 5,171,755, 5,304,325, 5,350,571, 5,393,524, and 5,403,575 and include those in which the perfluorocarbon compound is perfluorotributylamine, perfluorodecalin, perfluorooctylbromide, perfluorodichlorooctane, perfluorodecane, perfluorotripropylamine, perfluorotrimethylcyclohexane or other perfluorocarbon compounds. Further, mixtures of such perfluorocarbon compounds may be incorporated in the emulsions utilized in the practice of the invention.

As a specific example of a perfluorocarbon emulsion useful in the invention may be mentioned a perfluorodichlorooctane emulsion wherein the lipid coating thereof contains between approximately 50 to 99.5 mole percent lecithin, preferably approximately 55 to 70 to mole percent lecithin, 0 to 50 mole percent cholesterol, preferably approximately 25 to 45 mole percent cholesterol and approximately 0.5 to 10 mole percent biotinylated phosphatidylethanolamine, preferably approximately 1 to 5 mole percent biotinylated phosphatidylethanolamine. Other phospholipids such as phosphatidylserine may be biotinylated, fatty acyl groups such as stearylamine may be conjugated to biotin, or cholesterol or other fat soluble chemicals may be biotinylated and incorporated in the lipid coating for the lipid encapsulated particles. The preparation of an exemplary biotinylated perfluorocarbon for use in the practice of the invention is described hereinafter in accordance with known procedures.

When the lipid encapsulated particles are constituted by a liposome rather than an emulsion, such a liposome may be prepared as generally described in the literature

(see, for example, Kimelberg et al., *CRC Crit. Rev. Toxicol. 6*:25, 1978; Yatvin et al., *Medical Physics 9*:149, 1982). Liposomes are known to the art and generally comprise lipid materials including lecithin and sterols, egg phosphatidyl choline, egg phosphatidic acid, cholesterol and alpha-tocopherol.

Emulsifying agents, for example surfactants, may be used to facilitate the formation of emulsions and increase their stability. Typically, aqueous phase surfactants have been used to facilitate the formation of emulsions of fluorochemical liquids. A surfactant is any substance that contains both hydrophilic and a hydrophobic portions. When added to water or solvents, a surfactant reduces the surface tension. Preferred surfactants are phospholipids and cholesterol.

Any or a variety of lipid surfactants may be incorporated into the lipid monolayer preferably natural or synthetic phospholipids, but also fatty acids, cholesterols, lysolipids, sphingomyelins, tocopherols, glucolipids, stearylamines, cardiolipins, plasmalogens, a lipid with ether or ester linked fatty acids, polymerized lipids, and lipid conjugated polyethylene glycol. Other known surfactant additives such as PLURONIC F-68, HAMPOSYL L30 (W.R. Grace Co., Nashua, N.H.), sodium dodecyl sulfate, Aerosol 413 (American Cyanamid Co., Wayne, N.J.), Aerosol 200 (American Cyanamid Co.), LIPOPROTEOL LCO (Rhodia Inc., Mammoth, N.J.), STANDAPOL SH 135 (Henkel Corp., Teaneck, N.J.), FIZUL 10-127 (Finetex Inc., Elmwood Park, N.J.), and CYCLOPOL SBFA 30 (Cyclo Chemicals Corp., Miami, Fla.); amphoterics, such as those sold with the trade names: Deriphat.TM. 170 (Henkel Corp.), LONZAINE JS (Lonza, Inc.), NIRANOL C2N-SF (Miranol Chemical Co., Inc., Dayton, N.J.), AMPHOTERGE W2 (Lonza, Inc.), and AMPHOTERGE 2WAS (Lonza, Inc.); non-ionics, such as those sold with the trade names: PLURONIC F-68 (BASF Wyandotte, Wyandotte, Mich.), PLURONIC F-127 (BASF Wyandotte), BRIJ 35 (ICI Americas; Wilmington, Del.), TRITON X-100 (Rohm and Haas Co., Philadelphia, Pa.), BRIJ 52 (ICI Americas), SPAN 20 (ICI Americas), GENEROL 122 ES (Henkel Corp.), TRITON N-42 (Rohm and Haas Co.,), Triton.TM. N-101 (Rohm and Haas Co.,), TRITON X-405 (Rohm and tlaas Co.,), TWEEN 80 (ICI Americas), TWEEN 85 (ICI Americas), and BRIJ 56 (ICI Americas) and the like, may be used

alone or in combination in amounts of 0.10 to 5.0% by weight to assist in stabilizing the emulsions.

Fluorinated surfactants which are soluble in the fluorochemical liquid to be emulsified can also be used. Suitable fluorochemical surfactants include perfluorinated alkanoic acids such as perfluorohexanoic and perfluorooctanoic acids and amidoamine derivatives. These surfactants are generally used in amounts of 0.01 to 5.0% by weight, and preferably in amounts of 0.1 to 1.0%. Other suitable fluorochemical surfactants include perfluorinated alcohol phosphate esters and their salts; perfluorinated sulfonamide alcohol phosphate esters and their salts; perfluorinated alkyl sulfonamide alkylene quaternary ammonium salts; N,N-(carboxyl-substituted lower alkyl) perfluorinated alkyl sulfonamides; and mixtures thereof. As used herein, the term "perfluorinated" means that the surfactant contains at least one perfluorinated alkyl group.

Suitable perfluorinated alcohol phosphate esters include the free acids of the diethanolamine salts of mono- and bis(1H,1H,2H,2H-perfluoroalkyl)phosphates. The phosphate salts, available under the tradename ZONYL RP (E.I. Dupont de Nemours and Co., Wilmington, Del.), are converted to the corresponding free acids by known methods. Suitable perfluorinated sulfonamide alcohol phosphate esters are described in U.S. Pat. No. 3,094,547. Suitable perfluorinated sulfonamide alcohol phosphate esters and salts of these include perfluoro-n-octyl-N-ethylsulfonamidoethyl phosphate, bis(perfluoro-n-octyl-N-ethylsulfonamidoethyl) phosphate, the ammonium salt of bis(perfluoro-n-octyl-N-ethylsulfonamidoethyl)phosphate,bis(perfluorodecyl-N-ethylsulfonamidoethyl)-phosphate and bis(perfluorohexyl-N ethylsulfonamidoethyl)-phosphate. The preferred formulations use phosphatidylcholine, derivatized-phosphatidylethanolamine and cholesterol as the aqueous surfactant.

Lipid encapsulated emulsions may be formulated with cationic lipids in the surfactant layer that facilitate the adhesion of nucleic acid material to particle surfaces. Cationic lipids may include but are not limited to 1,2-Diacyl-3-Trimethylammonium-Propane (TAP), 1,2-Diacyl-3-Dimethylammonium-Propane (DAP), DC-Cholesterol (DC-Chol), Dimethyldioctadecylammonium Bromide (DDAB), 1,2-Diacyl-sn-Glycero-3-Ethylphosphocholine DOTMA, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-

trimethylammoium chloride; DOTAP, 1,2-dioleoyloxy-3-(trimethylammonio)propane; and DOTB,1,2-dioleoyl-3-(4'-trimethyl-ammonio)butanoyl-sn-glycerol may be used. In general the molar ratio of cationic lipid to non-cationic lipid in the lipid surfactant monolayer may be, for example, 1:1000 to 2:1, preferably, between 2:1 to 1:10, more preferably in the range between 1:1 to 1:2.5 and most preferably 1:1 (ratio of mole amount cationic lipid to mole amount non-cationic lipid, e.g., DPPC). A wide variety of lipids may comprise the non-cationic lipid component of the emulsion surfactant, particularly dipalmitoylphosphatidylcholine, dipalmitoylphosphatidyl-ethanolamine or dioleoylphosphatidylethanolamine in addition to those previously described. In lieu of cationic lipids as described above, lipids bearing cationic polymers such as polylysine or polyarginine may also be included in the lipid surfactant and afford binding of a negatively charged therapeutic, such as genetic material or analogues there of, to the outside of the emulsion particles.

The acoustic contrast substances of the present invention, which are preferably comprised of at least one perfluorocarbon, exhibit a temperature dependent reflectivity when bound to a targert. Perfluorocarbons have been reported to show a linear decrease in ultrasonic velocity with rise in temperature and a decrease in density over temperature ranges of as low as 10°C to as high as 50°C. (Narayana et al., *supra*, 1986). Thus, the temperature dependence of the nanoparticle emulsion or that of its constituent components, such as for example the preferred perfluorocarbon component, can be measured by determining ultrasound velocity of the emulsion or constituent component as is illustrated more fully below in the examples. It is believed that this measurement can also be used to predict the magnitude of change in reflectivity for a given perfluorocarbon component.

The ultrasound target may be an *in vivo* or *in vitro* target and, preferably, a biological material although the target need not be a biological material. The target may be comprised of a surface to which the acoustic contrast substance binds or a three dimensional structure in which the acoustic contrast substance penetrates and binds to portions of the target below the surface.

Preferably, a ligand is incorporated into the acoustic contrast substance to immobilize the acoustic contrast substance to the ultrasound target. The ligand may

be specific for a desired target to allow active targeting. Active targeting refers to ligand-directed, site-specific accumulation of agents to cells, tissues or organs by localization and binding to molecular epitopes, i.e., receptors, lipids, peptides, cell adhesion molecules, polysaccharides, biopolymers, and the like, presented on the surface membranes of cells or within the extracellular or intracellular matrix. A wide variety of ligands can be used including an antibody, a fragment of an antibody, a polypeptide such as small oligopeptide, a large polypeptide or a protein having three dimensional structure, a peptidomimetic, a polysaccharide, an aptamer, a lipid, a nucleic acid, a lectin or a combination thereof. The ligand specifically binds to a cellular epitope or receptor.

The term "ligand" as used herein is intended to refer to a small targeting molecule that binds specifically to another molecule of a biological target separate and distinct from the emulsion particle itself. The reaction does not require nor exclude a molecule that donates or accepts a pair of electrons to form a coordinate covalent bond with a metal atom of a coordination complex. Thus a ligand may be attached covalently for direct-conjugation or noncovalently for indirect conjugation to the surface of the acoustic particle surface.

Avidin-biotin interactions are extremely useful, noncovalent targeting systems that have been incorporated into many biological and analytical systems and selected *in vivo* applications. Avidin has a high affinity for biotin (10^{-15}M) facilitating rapid and stable binding under physiological conditions. Targeted systems utilizing this approach are administered in two or three steps, depending on the formulation. Typically, a biotinylated ligand, such as a monoclonal antibody, is administered first and "pretargeted" to the unique molecular epitopes. Next, avidin is administered, which binds to the biotin moiety of the "pretargeted" ligand. Finally, the biotinylated agent is added and binds to the unoccupied biotin-binding sites remaining on the avidin thereby completing the ligand-avidin-emulsion "sandwich". The avidin-biotin approach can avoid accelerated, premature clearance of targeted agents by the reticuloendothelial system secondary to the presence of surface antibody. Additionally, avidin, with four, independent biotin binding sites provides signal amplification and improves detection sensitivity.

As used herein, the term "biotin agent" or "biotinylated" with respect to conjugation to a biotin agent is intended to include biotin, biocytin and other biotin derivatives and analogs such as biotin amido caproate N-hydroxysuccinimide ester, biotin 4-amidobenzoic acid, biotinamide caproyl hydrazide and other biotin derivatives and conjugates. Other derivatives include biotin-dextran, biotin-disulfide-N-hydroxysuccinimide ester, biotin-6 amido quinoline, biotin hydrazide, *d*-biotin-N hydroxysuccinimide ester, biotin maleimide, *d*-biotin *p*-nitrophenyl ester, biotinylated nucleotides and biotinylated amino acids such as N, ε-biotinyl-l-lysine. The term "avidin agent" or "avidinized" with respect to conjugation to an avidin agent is intended to include avidin, streptavidin and other avidin analogs such as streptavidin or avidin conjugates, highly purified and fractionated species of avidin or streptavidin, and non-amino acid or partial-amino acid variants, recombinant or chemically synthesized avidin.

Targeting ligands may be chemically attached to the surface of acoustic particles by a variety of methods depending upon the nature of the particle surface. Conjugations may be performed before or after the emulsion particle is created depending upon the ligand employed. Direct chemical conjugation of ligands to proteinaceous agents often take advantage of numerous amino-groups (e.g. lysine) inherently present within the surface. Alternatively, functionally active chemical groups such as pyridyldithiopropionate, maleimide or aldehyde may be incorporated into the surface as chemical "hooks" for ligand conjugation after the particles are formed. Another common post-processing approach is to activate surface carboxylates with carbodiimide prior to ligand addition. The selected covalent linking strategy is primarily determined by the chemical nature of the ligand. Monoclonal antibodies and other large proteins may denature under harsh processing conditions; whereas, the bioactivity of carbohydrates, short peptides, aptamers, drugs or peptidomimetics often can be preserved. To ensure high ligand binding integrity and maximize targeted particle avidity flexible polymer spacer arms, e.g. polyethylene glycol or simple caproate bridges, can be inserted between an activated surface functional group and the targeting ligand. These extensions can be 10 nm or longer and minimize interference of ligand binding by particle surface interactions.

Monoclonal antibodies may also be used as site-targeting ligands directed to any of a wide spectrum of molecular epitopes including pathologic molecular epitopes. Immunoglobin-γ (IgG) class monoclonal antibodies have been conjugated to liposomes, emulsions and other microbubble particles to provide active, site-specific targeting. These proteins are symmetric glycoproteins (MW *ca.* 150,000 Daltons) composed of identical pairs of heavy and light chains. Hypervariable regions at the end of each of two arms provide identical antigen-binding domains. A variably sized branched carbohydrate domain is attached to complement-activating regions, and the hinge area contains particularly accessible interchain disulfide bonds that may be reduced to produce smaller fragments.

Bivalent $F(ab')_2$ and monovalent F(ab) fragments can be used as ligands and these are derived from selective cleavage of the whole antibody by pepsin or papain digestion, respectively. Elimination of the Fc region greatly diminishes the immunogenicity of the molecule, diminishes nonspecific liver uptake secondary to bound carbohydrate, and reduces complement activation and resultant antibody-dependent cellular toxicity. Complement fixation and associated cellular cytotoxicity can be detrimental when the targeted site must be preserved or beneficial when recruitment of host killer cells and target-cell destruction is desired (e.g. anti-tumor agents).

Most monoclonal antibodies are of murine origin and are inherently immunogenic to varying extents in other species. Humanization of murine antibodies through genetic engineering has lead to development of chimeric ligands with improved biocompatibility and longer circulatory half-lives. The binding affinity of recombinant antibodies to targeted molecular epitopes can be occasionally improved with selective site-directed mutagenesis of the binding idiotype.

Phage display techniques may be used to produce recombinant human monoclonal antibody fragments against a large range of different antigens without involving antibody-producing animals. In general, cloning creates large genetic libraries of corresponding DNA (cDNA) chains deducted and synthesized by means of the enzyme "reverse transcriptase" from total messenger RNA (mRNA) of human B lymphocytes. Immunoglobulin cDNA chains are amplified by PCR (polymerase chain

reaction) and light and heavy chains specific for a given antigen are introduced into a phagemid vector. Transfection of this phagemid vector into the appropriate bacteria results in the expression of an scFv immunoglobulin molecule on the surface of the bacteriophage. Bacteriophages expressing specific immunoglobulin are selected by repeated immunoadsorption/phage multiplication cycles against desired antigens (e.g., proteins, peptides, nuclear acids, and sugars). Bacteriophages strictly specific to the target antigen are introduced into an appropriate vector, (e.g., Escherichia coli, yeast, cells) and amplified by fermentation to produce large amounts of human antibody fragments with structures very similar to natural antibodies. Phage display techniques have permitted the production of unique ligands for targeting and therapeutic applications.

Polypeptides, like antibodies, may have high specificity and epitope affinity for use as vector molecules for targeted contrast agents. These may be small oligopeptides, having, for example, 5 to 10 amino acid, specific for a unique receptor sequences (such as, for example, the RGD epitope of the platelet GIIbIIIa receptor) or larger, biologically active hormones such as cholecystokinin. Smaller peptides potentially have less inherent immunogenicity than nonhumanized murine antibodies. Peptides or peptide (nonpeptide) analogues of cell adhesion molecules, cytokines, selectins, cadhedrins, Ig superfamily, integrins and the like may be utilized for targeted therapeutic delivery.

Asialoglycoproteins have been used for liver-specific applications due to their high affinity for asialoglycoproteins receptors located uniquely on hepatocytes. Asialoglycoproteins directed agents (primarily magnetic resonance agents conjugated to iron oxides) have been used to detect primary and secondary hepatic tumors as well as benign, diffuse liver disease such as hepatitis. The asialoglycoproteins receptor is highly abundant on hepatocytes, approximately 500,000 per cell, rapidly internalizes and is subsequently recycled to the cell surface. Polysaccharides such as arabinogalactan may also be utilized to localize agents to hepatic targets. Arabinogalactan has multiple terminal arabinose groups that display high affinity for asialoglycoproteins hepatic receptors.

Aptamers are high affinity, high specificity RNA or DNA-based ligands produced by *in vitro* selection experiments (SELEX: systematic evolution of ligands by exponential enrichment). Aptamers are generated from random sequences of 20 to 30 nucleotides, selectively screened by absorption to molecular antigens or cells, and enriched to purify specific high affinity binding ligands. To enhance *in vivo* stability and utility, aptamers are generally chemically modified to impair nuclease digestion and to facilitate conjugation with drugs, labels or particles. Other, simpler chemical bridges often substitute nucleic acids not specifically involved in the ligand interaction. In solution aptamers are unstructured but can fold and enwrap target epitopes providing specific recognition. The unique folding of the nucleic acids around the epitope affords discriminatory intermolecular contacts through hydrogen bonding, electrostatic interaction, stacking, and shape complementarity. In comparison with protein-based ligands, aptamers are stable, are more conducive to heat sterilization, and have lower immunogenicity. Aptamers are currently used to target a number of clinically relevant pathologies including angiogenesis, activated platelets, and solid tumors and their use is increasing. The clinical effectiveness of aptamers as targeting ligands for therapeutic emulsion particles may be dependent upon the impact of the negative surface charge imparted by nucleic acid phosphate groups on clearance rates. Previous research with lipid-based particles suggest that negative zeta potentials markedly decrease liposome circulatory half-life, whereas, neutral or cationic particles have similar, longer systemic persistence.

It is also possible to use what has been referred to as a "primer material" to couple specific binding species to the fluorchemical droplets as disclosed by Millbrath et al. (U.S. Patent No. 5,401,634) for certain *in vitro* applications. As used herein, "primer material" refers to any constituent or derivatized constituent incorporated into the emulsion lipid surfactant layer that could be chemically utilized to form a covalent bond between the particle and a targeting ligand or a component of the targeting ligand such as a subunit thereof.

Thus, the specific binding species (i.e. targeting ligand) may be immobilized on the encapsulating lipid monolayer by direct adsorption to the oil/aqueous interface or using a primer material. A primer material may be any surfactant compatible

compound incorporated in the particle to chemically couple with or adsorb a specific binding or targeting species. The preferred result is achieved by forming an emulsion with an aqueous continuous phase and a biologically active ligand adsorbed or conjugated to the primer material at the interface of the continuous and discontinuous phases. Naturally occurring or synthetic polymers with amine, carboxyl, mercapto, or other functional groups capable of specific reaction with coupling agents and highly charged polymers may be utilized in the coupling process. The specific binding species (e.g. antibody) may be immobilized on the fluorochemical emulsion particle surface by direct adsorption or by chemical coupling. Examples of specific binding species which can be immobilized by direct adsorption include small peptides, peptidomimetics, or polysaccharide-based agents. To make such an emulsion the specific binding species may be suspended or dissolved in the aqueous phase prior to formation of the emulsion. Alternatively, the specific binding species may be added after formation of the emulsion and incubated with gentle agitation at room temperature (25° C) in a pH 7.0 buffer (typically phosphate buffered saline) for 1.2 to 18 hours.

Where the specific binding species is to be coupled to a primer material, conventional coupling techniques may be used. The specific binding species may be covalently bonded to primer material with coupling agents using methods which are known in the art. Primer materials may include phosphatidylethanolamine (PE), N-caproylamine-PE, n-dodecanylamine, phosphatidylthioethanol,N-1,2-diacyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl)butyramide], 1,2-diacyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidomethyl)cyclohexane-carboxylate], 1,2-diacyl-sn-glycero-3-phosphoethanolamine-N-[3-(2-pyridyldithio)propionate], 1,2-diacyl-sn-glycero-3-phosphoethanolamine-N-[PDP(polyethylene glycol)2000], N-succinyl-PE, N-glutaryl-PE, N-dodecanyl-PE, N-biotinyl-PE, or N-caproyl-PE. Additional coupling agents use a carbodiimide such as1-ethyl-3-(3-N,N dimethylaminopropyl)carbodiimide hydrochloride or 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide methyl-p-toluenesulfonate. Other suitable coupling agents include aldehyde coupling agents having either ethylenic unsaturation such as acrolein, methacrolein, or 2-butenal, or having a plurality of aldehyde groups such as glutaraldehyde, propanedial or butanedial. Other coupling

agents include 2-iminothiolane hydrochloride, bifunctional N-hydroxysuccinimide esters such as disuccinimidyl subsrate, disuccinimidyl tartrate, bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone, disuccinimidyl propionate, ethylene glycolbis(succinimidyl succinate); heterobifunctional reagents such as N-(5-azido-2-nitrobenzoyloxy)succinimide, p-azidophenylbromide, p-azidophenylglyoxal,4-fluoro-3-nitrophenylazide, N-hydroxysuccinimidyl-4-azidobenzoate, m-maleimidobenzoyl N-hydroxysuccinimide ester, methyl-4-azidophenylglyoxal, 4-fluoro-3-nitrophenyl azide, N-hydroxysuccinimidyl-4-azidobenzoate hydrochloride, p-nitrophenyl 2-diazo-3,3,3-trifluoropropionate, N-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate, succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, succinimidyl 4-(p-maleimidophenyl)butyrate, N-succinimidyl(4-azidophenyldithio)propionate, N-succinimidyl 3-(2-pyridyldithio)propionate, N-(4-azidophenylthio)phthalamide; homobifunctional reagents such as 1,5-difluoro-2,4-dinitrobenzene, 4,4'-difluoro-3,3'-dinitrodiphenylsulfone, 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene, p-phenylenediisothiocyanate, carbonylbis(L-methionine p-nitrophenyl ester), 4,4'-dithiobisphenylazide, erythritolbiscarbonate and bifunctional imidoesters such as dimethyl adipimidate hydrochloride, dimethyl suberimidate, dimethyl 3,3'-dithiobispropionimidate hydrochloride and the like. Covalent bonding of a specific binding species to the primer material can be carried out with the above reagents by conventional, well-known reactions, for example, in the aqueous solutions at a neutral pH, at temperatures of less than 25 C for 1 hour to overnight.

The emulsions of the present invention may be prepared by various techniques. One method is sonication of a mixture of a fluorochemical liquid and an aqueous solution containing a suitable primer material and/or specific binding species. Generally, these mixtures include a surfactant. Cooling the mixture being emulsified, minimizing the concentration of surfactant, and buffering with a saline buffer will typically maximize both retention of specific binding properties and the coupling capacity of the primer material. These techniques provide excellent emulsions with high activity per unit of absorbed primer material or specific binding species.

When high concentrations of a primer material or specific binding species coated on lipid emulsions, the mixture should be heated during sonication and have a

relatively low ionic strength and moderate to low pH. Too low an ionic strength, too low a pH or too much heat may cause some degradation or loss of all of the useful binding properties of the specific binding species or the coupling capacity of the "primer" material. Careful control and variation of the emulsification conditions can optimize the properties of the primer material or the specific binding species while obtaining high concentrations of coating.

Carbohydrate-bearing lipids may be employed for in vivo targeting, as described in U.S. Pat. No. 4,310,505, the disclosures of which are hereby incorporated herein by reference, in their entirety.

An alternative method of making the emulsions involves directing high pressure streams of mixtures containing the aqueous solution, a primer material or the specific binding species, the fluorocarbon liquid and a surfactant (if any) so that they impact one another to produce emulsions of narrow particle size and distribution. The MICROFLUIDIZER apparatus (Microfluidics, Newton, Mass.) can be used to make the preferred emulsions. The apparatus is also useful to post-process emulsions made by sonication or other conventional methods. Feeding a stream of emulsion droplets through the MICROFLUIDIZER apparatus yields formulations small size and narrow particle size distribution.

Emulsifying and/or solubilizing agents may also be used in conjunction with emulsions. Such agents include, but are not limited to, acacia, cholesterol, diethanolamine, glyceryl monostearate, lanolin alcohols, lecithin, mono- and di-glycerides, mono-ethanolamine, oleic acid, oleyl alcohol, poloxamer, peanut oil, palmitic acid, polyoxyethylene 50 stearate, polyoxyl 35 castor oil, polyoxyl 10 oleyl ether, polyoxyl 20 cetostearyl ether, polyoxyl 40 stearate, polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 80, propylene glycol diacetate, propylene glycol monostearate, sodium lauryl sulfate, sodium stearate, sorbitan mono-laurate, sorbitan mono-oleate, sorbitan mono-palmitate, sorbitan monostearate, stearic acid, trolamine, and emulsifying wax. All lipids with perfluoro fatty acids as a component of the lipid in lieu of the saturated or unsaturated hydrocarbon fatty acids found in lipids of plant or animal origin may be used. Suspending and/or viscosity-increasing agents that may be used with emulsions include, but are not limited to, acacia, agar, alginic acid,

aluminum mono-stearate, bentonite, magma, carbomer 934P, carboxymethylcellulose, calcium and sodium and sodium 12, carrageenan, cellulose, dextrin, gelatin, guar gum, hydroxyethyl cellulose, hydroxypropyl methylcellulose, magnesium aluminum silicate, methylcellulose, pectin, polyethylene oxide, polyvinyl alcohol, povidone, propylene glycol alginate, silicon dioxide, sodium alginate, tragacanth, and xanthum gum.

Useful emulsions may have a wide range of nominal particle diameters, e.g., from as small as about 0.01 μ to as large as 10 μ, preferably about 0.1 to about 0.5 μ in diameter. The emulsion particle sizes can be controlled and varied by modifications of the emulsification techniques and the chemical components. Small size particles are preferred because they circulate longer and tend to be more stable than larger particles.

Targeted therapeutic emulsions may incorporate bioactive agents (e.g drugs, prodrugs, genetic materials, radioactive isotopes, or combinations thereof) in their native form or derivatized with hydrophobic or charged moieties to enhance incorporation or adsorption to the ligand targeted particle. The bioactive agent may be a prodrug, including the prodrugs described, for example, by Sinkyla et al., J. Pharm. Sci., 64:181-210 (1975), in U.S. application Ser. No. 08/851,780 filed May 6, 1997, and in U.S. application Ser. No. 08/887,215 filed Jul. 2, 1997, the disclosures of which are hereby incorporated by reference herein in their entirety.

Such therapeutics may also include, but are not limited to antineoplastic agents, including platinum compounds (e.g., spiroplatin, cisplatin, and carboplatin), methotrexate, fluorouracil, adriamycin, mitomycin, ansamitocin, bleomycin, cytosine arabinoside, arabinosyl adenine, mercaptopolylysine, vincristine, busulfan, chlorambucil, melphalan (e.g., PAM, L-PAM or phenylalanine mustard), mercaptopurine, mitotane, procarbazine hydrochloride dactinomycin (actinomycin D), daunorubicin hydrochloride, doxorubicin hydrochloride, taxol, plicamycin (mithramycin), aminoglutethimide, estramustine phosphate sodium, flutamide, leuprolide acetate, megestrol acetate, tamoxifen citrate, testolactone, trilostane, amsacrine (m-AMSA), asparaginase (L-asparaginase) Erwina asparaginase, interferon α-2a, interferon α-2b, teniposide (VM-26), vinblastine sulfate (VLB), vincristine sulfate, bleomycin, bleomycin sulfate, methotrexate, adriamycin, arabinosyl,

hydroxyurea,procarbazine, dacarbazine, mitotic inhibitors such as etoposide and other vinca alkaloids; radiopharmaceuticals such as but not limited to radioactive iodine, samarium, strontium cobalt, yittrium and the like; protein and nonprotein natural products or analogues/mimetics thereof including hormones such as but not limited to growth hormone, somatostatin, prolactin, thyroid, steroids, androgens, progestins, estrogens and antiestrogens; analgesics including but not limited to antirheumatics, such as auranofin, methotrexate, azathioprine, sulfazalazine, leflunomide, hydrochloroquine, and etanercept; muscle relaxants such as baclofen, dantrolene, carisoprodol, diazepam, metaxalone, cyclobenzaprine, chlorzoxazone, tizanidine; narcotic agonists such as codeine, fentanyl, hydromorphone, lleavorphanol, meperidine, methadone, morphine, oxycodone, oxymorphone, propoxyphene; narcotic agonist-antagonists such as buprenorphine, butorphanol, dezocine, nalbuphine, pentazocine; narcotic antagonists such as nalmefene and naloxone, other analgesics including ASA, acetominophen, tramadol, or combinations thereof; nonsteroidal anti-inflammatories including but not limited to celecoxib, diclofenac, diflunisal, etodolac, fenoprofen, flurbiprofen, ibuprofen, indomethacin, ketoprofen, ketorolac, naproxen, oxaproxen, rofecoxib, salisalate, suldindac, tolmetin; anesthetic and sedatives such as etomidate, fentanyl, ketamine, methohexital, propofol, sufentanil, thiopental, and the like; neuromuscular blockers such as but not limited to pancuronium, atracurium, cisatracurium, rocuronium, succinylcholine, vercuronium; antimicrobials including aminoglycosides, antifungal agents including amphotericin B, clotrimazole, fluconazole, flucytosine, griseofulvin, itraconazole, ketoconazole, nystatin, and terbinafine; anti-helmintics; antimalarials, such as chloroquine, doxycycline, mefloquine, primaquine, quinine; antimycobacterial including dapsone, ethambutol, ethionamide, isoniazid, pyrazinamide, rifabutin, rifampin, rifapentine; antiparasitic agents including albendazole, atovaquone, iodoquinol, ivermectin,mebendazole, metronidazole, pentamidine, praziquantel, pyrantel, pyrimethamine, thiabendazole; antiviral agents including abacavir, didanosine, lamivudine, stavudine, zalcitabine, zidovudine as well as protease inhibitors such as indinavir and related compounds, anti-CMV agents including but not limited to cidofovir, foscarnet, and ganciclovir; antiherpetic agents including amatadine, rimantadine, zanamivir; interferons, ribavirin, rebetron; carbapenems, cephalosporins, fluoroquinones, macrolides,

penicillins, sulfonamides, tetracyclines, and other antimicrobials including aztreonam, chloramphenicol, fosfomycin, furazolidone, nalidixic acid, nitrofurantoin, vancomycin. and the like; nitrates, antihypertensives including diuretics, beta blockers, calcium channel blockers, angiotensin converting enzyme inhibitors, angiotensin receptor antagonists, antiadrenergic agents, anti-dysrhythmics, antihyperlipidemic agents, antiplatelet compounds, pressors, thrombolytics, acne preparations, antipsoriatics; corticosteroids; androgens, anabolic steroids, bisphosphonates; sulfonoureas and other antidiabetic agents; gout related medicants; antihistamines, antitussive, decongestants, and expectorants; antiulcer medicants including antacids, 5-HT receptor antagonists, H2-antagonists, bismuth compounds, proton pump inhibitors, laxatives, octreotide and its analogues/mimetics; anticoagulants; immunization antigens, immunoglobins, immunosuppressive agents; anticonvulsants, 5-HT receptor agonists, other migraine therapies; parkinsonian agents including anticholinergics, and dopaminergics; estrogens, GnRH agonists, progestins, estrogen receptor modulators, tocolytics, uterotnics,thyroid agents such as iodine products and anti-thyroid agents; blood products such as parenteral iron, hemin, hematoporphyrins and their derivatives.

Genetic material, includes, for example, nucleic acids, RNA and DNA, of either natural or synthetic origin, including recombinant RNA and DNA and antisense RNA and DNA; hammerhead RNA, ribozymes, hammerhead ribozymes, antigene nucleic acids, both single and double stranded RNA and DNA and analogs thereof, ribooligonucleotides, antisense ribooligonucleotides, deoxyribooligonucleotides, and antisense deoxyribooligonucleotides. Other types of genetic material that may be used include, for example, genes carried on expression vectors such as plasmids, phagemids, cosmids, yeast artificial chromosomes, and defective or "helper" viruses, antigene nucleic acids, both single and double stranded RNA and DNA and analogs thereof, such as phosphorothioate and phosphorodithioate oligodeoxynucleotides. Additionally, the genetic material may be combined, for example, with proteins or other polymers.

As we have previously described, the emulsion nanoparticles may incorporate on the particle paramagnetic or super paramagnetic elements including but not limited

to gadolinium, magnesium, iron, manganese in their native or in a chemically complexed form. Similarly, radioactive nuclides including positron-emitters, gamma-emitters, beta-emitters, alpha-emitters in their native or chemically-complexed form may be included on or in the particles. Adding of these moieties permits the additional use of other clinical imaging modalities such as magnetic resonance imaging, positron emission tomography, and nuclear medicine imaging techniques in conjunction with temperature enhanced ultrasonic imaging. Moreover, the inclusion of metal ions in or on the formulation may be utilized as "seeds" to augment or implement local hyperthermia.

In addition, optical imaging, which refers to the production of visible representations of tissue or regions of a patient produced by irradiating those tissues or regions of a patient with electromagnetic energy in the spectral range between ultraviolet and infrared, and analyzing either the reflected, scattered, absorbed and/or fluorescent energy produced as a result of the irradiation, may be combined with the enhanced acoustic reflectivity of temperature-dependent targeted emulsions. Examples of optical imaging include, but are not limited to, visible photography and variations thereof, ultraviolet images, infrared images, fluorimetry, holography, visible microscopy, fluorescent microscopy, spectrophotometry, spectroscopy, fluorescence polarization and the like.

Photoactive agents, i.e. compounds or materials that are active in light or that responds to light, including, for example, chromophores (e.g., materials that absorb light at a given wavelength), fluorophores (e.g., materials that emit light at a given wavelength), photosensitizers (e.g., materials that can cause necrosis of tissue and/or cell death in vitro and/or in vivo), fluorescent materials, phosphorescent materials and the like, that may be used in diagnostic or therapeutic applications. "Light" refers to all sources of light including the ultraviolet (UV) region, the visible region and/or the infrared (IR) region of the spectrum. Suitable photoactive agents that may be used in the present invention have been described by others (Unger et al 6,123,923) are incorporated by reference herein and include but are not limited to, for example, fluoresceins, indocyanine green, rhodamine, triphenylmethines, polymethines, cyanines, fullerenes, oxatellurazoles, verdins, rhodins, perphycenes, sapphyrins,

rubyrins, cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoate, cholesteryl 12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-dodecanate, cholesteryl cis-parinarate, cholesteryl 3-((6-phenyl)-1,3,5-hexatrienyl)phenyl-proprionate, cholesteryl 1-pyrenebutyrate, cholesteryl 1-pyrenedecanoate, cholesteryl 1-pyrenehexanoate, 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3.be ta.-ol, 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3.be ta.-yl cis-9-octadecenoate, 1-pyrenemethyl3-(hydroxy-22,23-bisnor-5-cholenate, 1-pyrene-methyl 3.beta.-(cis-9-octadecenoyloxy)-22,23-bisnor-5-cholenate, acridine orange 10-dodecyl bromide, acridine orange 10-nonyl bromide, 4-(N,N-dimethyl-N-tetradecylammonium)-methyl-7-hydroxycoumarin) chloride, 5-dodecanoylaminofluorescein, 5-dodecanoyl-aminofluorescein-bis-4,5-dimethoxy-2-nitrobenzyl ether, 2-dodecylresorufin, fluorescein octadecyl ester, 4-heptadecyl-7-hydroxycoumarin, 5-hexadecanoylaminoeosin, 5-hexadecanoylaminofluorescein,5-octadecanoylaminofluorescein, N-octadecyl-N'-(5-(fluoresceinyl))thiourea, octadecyl rhodamine B chloride, 2-(3-(diphenylhexatrienyl)-propanoyl)-1-hexadecanoyl-sn-glycero- 3-phosphocholine, 6-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic acid, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate, 12-(9-anthroyloxy)oleic acid, 5-butyl-4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-3-nonanoic acid, N-(lissamine.TM. rhodamine B sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt, phenylglyoxal monohydrate, naphthalene-2,3-dicarboxaldehyde, 8-bromomethyl-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indace ne, o-phthaldialdehyde, lissamine.TM. rhodamine B sulfonyl chloride, 2',7'-difluorofluorescein, 9-anthronitrile, 1-pyrenesulfonyl chloride, 4-(4-(dihexadecylamino)-styryl)-N-methylpyridinium iodide, chlorins, such as chlorin, chlorin e6, bonellin, mono-L-aspartyl chlorin e6, mesochlorin, mesotetraphenylisobacteriochlorin, and mesotetraphenylbacteriochlorin, hypocrellin B, purpurins, such as octaethylpurpurin, zinc(II) etiopurpurin, tin(IV) etiopurpurin and tin ethyl etiopurpurin, lutetium texaphyrin, photofrin, metalloporphyrins, protoporphyrin IX, tin protoporphyrin, benzoporphyrin, haematoporphyrin, phthalocyanines, naphthocyanines, merocyanines, lanthanide complexes, silicon

phthalocyanine, zinc phthalocyanine, aluminum phthalocyanine, Ge octabutyoxyphthalocyanines, methyl pheophorbide-.alpha.-(hexyl-ether), porphycenes, ketochlorins, sulfonated tetraphenylporphines, .delta.-aminolevulinic acid, texaphyrins, including, for example, 1,2-dinitro-4-hydroxy-5-methoxybenzene, 1,2-dinitro-4-(1-hydroxyhexyl)oxy-5-methoxybenzene, 4-(1-hydroxyhexyl)oxy-5methoxy-1,2-phenylenediamine, and texaphyrin-metal chelates, including the metals Y(III), Mn(II), Mn(III), Fe(II), Fe(III) and the lanthanide metals Gd(III), Dy(III), Eu(III), La(III), Lu(III) and Tb(III), chlorophyll, carotenoids, flavonoids, bilins, phytochromes, phycobilins, phycoerythrins, phycocyanines, retinoic acids, retinoins, retinates, or combinations of any of the above.

One skilled in the art will readily recognize or can readily determine which of the above compounds are, for example, fluorescent materials and/or photosensitizers. LISSAMINE. is the trademark for N-ethyl-N-[4-[[4-[ethyl [(3-sulfophenyl)methyl]-amino]phenyl](4-sulfophenyl)-methylene]-2,5-cyclohexadien-1-ylidene]-3-sulfobenzene-methanaminium hydroxide, inner salt, disodium salt and/or ethyl[4-[p[ethyl(m-sulfobenzyl)amino]-.alpha.-(p-sulfophenyl)benzylidene]- 2,5-cyclohexadien-1-ylidene](m-sulfobenzyl)ammonium hydroxide inner salt disodium salt (commercially available from Molecular Probes, Inc., Eugene, Oreg.). Other suitable photoactive agents for use in the present invention include those described in U.S. Pat. No. 4,935,498, the disclosure of which is hereby incorporated by reference herein in its entirety, such as a dysprosium complex of 4,5,9,24-tetraethyl-16-(1-hydroxyhexyl)oxy-17 methoxypentaazapentacyclo-(2 0.2.1.1.sup.3,6.1. sup.8,11.0.sup.14,19)-heptacosa-1,3,5,7,9,11(27),12,14,16,18,20,22(25),23-tridecaene and dysprosium complex of 2-cyanoethyl-N,N-diisopropyl-6-(4,5,9,24-tetraethyl-17-methoxypentaazapent acyclo-(20.2.1.1 .sup.3,6.1.sup.8,11.0.sup.14,19)-heptacosa-1,3,5,7,9,11(27), 12,14,16,18,20,22(25),23-tridecaene-16-(1-oxy)hexylphosphoramidite.

In addition, certain ligands, such as, for example, antibodies, peptide fragments, or mimetics of a biologically active ligand may contribute to the inherent therapeutic effects, either as an antagonistic or agonistic, when bound to specific epitopes. As an example, antibody against $\alpha_v\beta_3$ integrin on neovascular endothelial

cells has been shown to transiently inhibit growth and metastasis of solid tumors. The efficacy of therapeutic emulsion particles directed to the $\alpha_v\beta_3$ integrin may result from the improved antagonistic action of the targeting ligand in addition to the effect of the therapeutic agents incorporated and delivered by particle itself.

Changes in temperature of the acoustic imaging substance bound to the target can involve increases or decreases in temperature. In embodiments in which the temperature of the acoustic imaging substance is increased, an energy source is used to increase the energy and an increase in acoustic reflectivity is measured.

Local hyperthermia may be induced at the site of targeted nanoparticle emulsions by a variety of modalities including but not limited to ultrasound, shortwave, microwave, magnetic radiation, electromagnetic energies or combination thereof. Such energy may be applied noninvasively by external systems or more invasively by catheter systems. Yang et al. (*Med Biol Eng Comput 17*:518-24, 1979) have shown that microwaves (e.g. 2500 MHz) provide excellent superficial heating to the skin, 900 MHz radiation induces pronounced temperature rises in the musculature and shortwaves at 27 MHz produce a wide plateau of elevated temperature in the muscle layers. These investigators have shown how wave frequency, power, ambient conditions, vasodilation and core boundary conditions can be varied to control local hyperthermia. Others have report the use of ferrimagnetic resonance of a ferrite-impregnated medium as the heating target which is placed internally and heated externally by radiated microwaves. This increased the depth of heating by up to 50% versus nonresonance techniques.

Minimally invasive thermal therapy is a currently used cancer treatment for treating solid tumors and the procedure can also provide the local hyperthemia for temperature-dependent acoustic contrast enhancement of ligand-targeted emulsions. Such approaches impart high temperatures over short time-frames (from microseconds to minutes). Interstitial heating localize the target tissue volume and minimize the effect of heating on surrounding normal tissues. Interstitial heating energy is typically delivered by laser light, microwaves, or ultrasound. The choice of energy source depends on the target site, applicator geometry, and blood perfusion to the site. The decrease of energy with distance due to applicator geometry is more important for

targets close to the applicator while the fall-off of energy due to attenuation is more important further from the energy source. Thus, laser light, which is highly scattered in tissues, is appropriately applied to targets close to the applicator and ultrasound or microwaves are better suited for heating deeper structures (Skinner et al., *Phys Med Biol 43*:3535-47, 1998).

The heating effect is believed to is believed to require intensities that are preferably greater than 0.1 W/cm2 (typical ultrasound imaging fields). Also, the prefered intensity level is less than those used for high intensity focused ultrasound (such as, for example, approximately 2000 W/cm2). Additional preferences are that nondestructive pulses and intensity levels be used to leave the tissue itself unharmed. It is believed that high intensity but short pulse durations will also be useful for this application in the range of microseconds to milliseconds, depending on transducer characteristics, depth of tissue interrogated, tissue attenuation,beam dispersion and other physical features. These parameters are themselves distinguishable from more prevalent high intensity focused ultrasound techniques for therapeutic ultrasound uses.

In other instances, the methods of the present invention involve decreasing the temperature of the acoustic imaging substance and measurement of the decrease in reflectivity. The decrease in temperature can be produced by an energy absorbing component such as a cryogenic device for use in cryotherapy. Cryotherapy, which is also sometimes referred to as cryosurgery, is well known in the art involving the use of a liquid nitrogen or liquid argon in a probe as an energy absorber such that the extreme cold kills cancer cells contacted by the probe. (see for example Lee et al., *Urology 54*:135-40, 1999). The methods of the present invention provide an approach for targeting of the energy absorber based upon the decrease in acoustic reflectivity of the acoustic imaging substance bound to the target tissue as well as providing an approach for monitoring the decrease in temperature.

In certain embodiments, the methods of the present invention involve the measurement of the acoustic reflectivity of the acoustic imaging substance upon changing the temperature of the acoustic imaging substance compared to the acoustic reflectivity in absence of the energy source or the energy absorber which changes the temperature of the acoustic imaging substance. The measurement in absence of the

energy source or energy absorber can be performed either before or after the energy change. Thus it is possible to measure acoustic reflectivity under control conditions prior to and then immediately after temperature change. Alternatively, the acoustic reflectivity can be measured upon achieving the energy change and then some time later after the energy change has dissipated to return the target to a temperature approaching that prior to the energy change.

The measurement of the difference in acoustic reflectivity can be represented in a number of ways, for example, as a digital numeric representation of the reflectivity difference, as a differential two or three dimensional image, as a colorized differential image and the like.

The present invention also includes devices for performing the methods. The devices measure changes in temperature of a target to which a temperature-sensitive acoustic imaging substance is bound. The device comprises a component configured to change the temperature of the acoustic imaging substance, an ultrasound source configured to transmit acoustic energy to the target, an ultrasound detecting component configured to measure acoustic reflectivity of the surface and a comparator which determines acoustic reflectivity of the target upon changing temperature relative to acoustic reflectivity of the target in absence of changing temperature. The ultrasound transmitting component and ultrasound detecting component are preferably comprised of at least one ultrasound piezoelectric transducer. The temperature changing component can be an energy source, such as a source for ultrasound, shortwave, microwave, magnetic radiation, electromagnetic energy or an energy absorber such as a cryogenic component comprising circulated liquid nitrogen or liquid argon. Preferably, the energy changing component is in the form of a probe.

The comparator component of the device provides the differential measurement in the absence and presence of the temperature change produced by the energy changing component. In one embodiment, the comparator comprises an image processor for producing a differential image based upon a subtraction of the acoustic reflectivity images produced in absence and presence of the energy change. The image processor may include an image frame storage component and/or electronic

components such as computer hardware and software components to produce the subtraction image.

Preferred embodiments of the invention are described in the following examples. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the examples.

EXAMPLE 1

This example illustrates the measurement of temperature dependence of ultrasound velocity in perfluorooctane, perfluorodichlorooctane and perfluorooctylbromide.

Ultrasound velocities were determined using a 25 MHz, Panametrics V324 spherically focused transducer. Measurements were made for perfluorooctane, perfluorodichlorooctane or perfluorooctylbromide at discrete temperatures by placing 8 mL of fluorocarbon in a sealed, vertically mounted sample chamber in heated water bath. The back of the chamber consisted of a stainless steel reflector, which extended past the fully enclosed well to allow for water-path and sample-path measurements. The chamber was mounted so that the stainless steel reflector was perpendicular to the insonifying beam.

The times of flight from the transducer to front wall of the chamber and from the transducer to the stainless steel plate were determined for nine independent locations over the sample. The speeds of sound were then averaged together for each temperature. The temperature was changed by two-degree increments from 25 to 47 C by heating the surrounding water bath and allowing time for the sample to reach equilibrium (typically 20 to 25 minutes). Speed of sound was calculated using a previously published algorithm.(Kuo et al., *J. Acoust. Soc. Am. 88*:1679-82, 1990)

Speed of sound measurements are summarized in Figure 1. As has been previously reported for other liquid perfluorocarbons, the speed of sound showed a linear decrease with increasing temperature for each of the perfluorocarbons.

EXAMPLE 2

This example illustrates the preparation of a biotinylated microemulsion for avidin-biotin targeting.

A biotinylated emulsion was prepared by incorporating biotinylated phosphatidylethanolamine into the outer lipid monolayer of a perfluorocarbon microemulsion. The microemulsion was prepared containing perfluorooctane (40% w/v, 3M), vegetable oil (2% w/v) a surfactant co-mixture (2.0%, w/v) and glycerin (1.7%, w/v) in water as follows. The surfactant co-mixture was prepared by dissolving 64 mole% lecithin (Pharmacia, Inc), 35 mole% cholesterol (Sigma Chemical Co.) and 1 mole% N-(6-(biotinoyl)amino) hexanoyl)-dipalmitoyl-L-alpha-phosphatidyl-ethanolamine, Pierce, Inc.) in chloroform. The chloroform-lipid mixture was evaporated under reduced pressure, dried in a 50°C vacuum oven overnight and dispersed into water by sonication. The suspension was then transferred into a blender cup (Dynamics Corporation of America) with the fluorocarbon, vegetable oil, glycerin and distilled, deionized water and emulsified for 30 to 60 seconds. The emulsified mixture was transferred to a MICROFLUIDICS emulsifier (Microfluidics Co.) and continuously processed at 20,000 PSI for three minutes. The completed emulsion was vialed, blanketed with nitrogen and sealed with stopper crimp seal until use. Particle sizes were determined in triplicate at 37°C with a laser light scattering submicron particle size analyzer (Malvern Zetasizer 4, Malvern Instruments Ltd, Southborough, MA)., which indicated a narrow size distribution with average particle diameter less than 400 nm.

EXAMPLE 3

This example illustrates a method which can be used to prepare an emulsion in which the nanoparticles are conjugated with an F(ab) fragment.

Targeting of emulsions can be achieved by direct chemical conjugation of an antibody to the nanoparticle through a primer material incorporated into the lipid monolayer. The perfluorocarbon nanoparticle contrast agent is prepared as described in Example 1.

F(ab) fragments are generated and isolated using an immunopure F(ab) preparation kit (Pierce, Rockford, IL). Briefly, IgG is dialyzed into 20mM phosphate/10mM EDTA buffer (pH 7.0), concentrated to 20 mg/ml and digested by immobilized papain. Solubilized F(ab) is purified from Fc fragments and undigested IgG protein using a protein A column. F(ab) fragments are purified from excess cysteine using a G25-150 column and deoxygenated phosphate buffer (pH 6.7). Fraction identity is confirmed by routine SDS-PAGE procedures.

F(ab) fractions are pooled and combined with the primer-derivatized emulsion (1-2 mg F(ab)/ml of emulsion). The mixture is adjusted to pH 6.7, sealed under nitrogen and allowed to react overnight at ambient temperatures with gentle, continuous mixing. The mixture may be subsequently dialyzed with a 300,000 MWCO Spectra/Por DispoDialyzer (Laguna Hills, CA) against 10mM phosphate buffer (pH 7.2) to remove unconjugated F(ab) fragments. The final emulsion is vialed under nitrogen and stored at 4° C until use. A nonspecific control emulsion may be prepared using the control, irrelevant IgG F(ab) fragments in the above protocol.

EXAMPLE 4

This example illustrates the temperature-dependent targeting of nitrocellulose membranes using microemulsion ultrasound contrast agent bound to the target with avidin-biotin conjugation.

The ultrasonic data acquisition set-up and analysis to measure acoustic reflectance backscatter was as follows. A 25MHz, spherically focused transducer (0.63 mm diameter, 2.54mm focal length, Panametrics V324) was mounted on a gantry consisting of three orthogonal sleds. The transducer was translated in a raster scan format by a computer controlled motion controller (Aerotech Unidex 12) with 100 μm resolution. The pulses sent to the motor from the motion controller were counted in a digital counter (National Instruments PCI-1200) and then a trigger was generated for a digital delay generator (Stanford Research Systems DG535). The delay generator then sent a trigger for the pulser (Panametrics 5900) and for the digitizing oscilloscope (Hewlett-Packard 54510B), as well as a delayed trigger for the real time digitizer (Tektronix RTD720A). Traces representing the backscattered

ultrasonic wave were captured on the fly as the transducer was scanned over the surface of the clot in a 68 x 68 (6.8 mm x 6.8 mm) grid at 100 μm resolution. The traces were then transferred from the real time digitizer to the controlling computer (Apple Power Macintosh 7300) over GPIB for image reconstruction and data storage. Acquisition typically took about 4 minutes per scan.

The sample chamber consisted of a fully enclosed well with an acoustic aperture to allow insonification of the sample. The chamber was attached through two ports to silicone tubing (Masterflex Platinum, I.D.=1/8") that allowed perfusion of the contrast agent over the sample. A roller pump (Masterflex, Cole-Parmer Inc.) was used to drive the flow at a rate of 20mL/min. The flow system was filled with 20 mL of 50nM phosphate buffer. The sample chamber and enclosed sample were positioned vertically so that no passive settling of the contrast agent could occur. After initial location of the sample, a bolus of 100μL of the contrast agent was delivered through an injection port and ultrasonic monitoring was performed initially and after 60 minutes of exposure. The chamber and tubing were then flushed with phosphate buffer.

After confirmation of successful targeting of the contrast agent by imaging, the temperature was varied in 5° C increments from 27 to 47° C using an immersion heater controlled by a temperature regulator (DigiSense, Cole-Parmer Inc.). The entire water bath was placed on top of a magnetic stirrer plate to allow for adequate mixing and homogenous temperature distribution throughout the bath. At each temperature point, the focus of the transducer was determined by observing the reflection from a steel plate. The front wall of the sample was then placed at the focus of the transducer.

The reflected ultrasonic signals were full-wave rectified and used to render a peak-detected c-scan image so that a user-defined region of interest could be drawn around the clot or nitrocellulose sample. The signals representing the reflection of the interrogating wave of ultrasound from the surface of the sample were isolated with a rectangular windowing function. The placement of the window was carefully controlled in the case of the thin nitrocellulose samples by an automatic algorithm that placed the end of the window midway between the front and back wall echo of the

nitrocellulose paper. The isolated signal was then fast Fourier transformed and the average power over the usable bandwidth (17 to 35 MHz, as determined by 10dB down points) was calculated in the logarithmic domain. This "integrated power" was then sorted for all of the points in the region of interest and the brightest 100 points were retained for analysis. The integrated power determined at every point in the scan was also used to render images of the change in ultrasonic enhancement of the clot. The frequency-dependent reflection enhancement was averaged for the 100 brightest points and then normalized by subtracting the reflection enhancement for the control scan. This process was performed for each sample.

Nitrocellulose membranes were prepared as follows for avidin-biotin targeting of temperature-dependent targeted emulsions in vitro. Flat nitrocellulose membranes were prepared for contrast binding with a diaminohexane spacer and activated with glutaraldehyde for protein conjugation. Nitrocellulose discs (2.5 cm diameter) were immersed in 1,6 diaminohexane (2.5% w/v, pH 11.9) for 60 minutes under constant rotary agitation. The membranes were next washed under constant agitation for approximately 12 hours in 1M acetic acid followed by 12 hours in ultrapure water with several changes of each medium. The diaminoalkane-modified nitrocellulose membranes were then exposed to 1% glutaraldehyde in 0.5 M $NaHCO_3$ / Na_2CO_3, pH 10, for 15 minutes followed by a three hour wash in several changes of ultrapure water. The diaminohexane-modified, glutaraldehyde-activated nitrocellulose membranes were stored dry at 4° C until use.

Avidin (50 μg) dissolved in 0.1 M phosphate buffered saline (PBS) (pH 7.2-7.4) was spotted and air-dried dropwise onto the center of each membrane with a microliter syringe and allowed to dry. Next, each membrane was washed with three, five-minute changes of PBS-0.1% Tween 20. Dehydrated milk powder suspended in PBS-0.1% Tween 20 was used to block glutaraldehyde activated protein binding sites left unoccupied after the application of avidin for 20 minutes. Excess protein was removed with three, five minute isotonic, PBS washes.

Five avidin-derivatized and five control nitrocellulose discs were utilized for exposure to biotinylated perfluorooctane particles. After confirmation of successful targeting of the contrast agent by acoustic imaging, the targeted sample temperature

was varied in 5° C increments from 27 to 47° C using an immersion heater controlled by a temperature regulator (DigiSense, Cole-Parmer Inc.). The entire water bath was placed on top of a magnetic stirrer plate to allow for adequate mixing and homogenous temperature distribution throughout the bath. At each temperature point, the focus of the transducer was determined by observing the reflection from a steel plate. The front wall of the sample was then placed at the focus of the transducer.

Figure 2 demonstrates the change of ultrasonic reflected power with temperature for targeted and control nitrocellulose membranes. The membranes were spotted with a single drop of avidin resulting in the almost perfectly circular feature. The pictures shows the change in reflected enhancement for the transition from the baseline temperature of 27 C to each individual temperature points. Darker areas represent areas of larger changes in enhancement and can be seen to increase substantially for targeted nitrocellulose in comparison to the control nitrocellulose.

The 100 brightest sites for each nitrocellulose membrane sample were selected to determine the average frequency-dependent reflection enhancement. Each curve was then normalized to the respective reflection enhancement from the control nitrocellulose membrane at the same temperature.

The progressive increase in ultrasonic backscatter enhancement with temperature was further quantified in Figure 3 with the bandwidth (17 to 34 MHz) limited average. The increased impedance mismatch between the bound nanoparticles and the substrate that occurs with increased temperature results in improved reflection enhancement from targeted substrates. The correlation between contrast enhancement and temperature for nitrocellulose (R=0.95) is excellent. Figures 3 quantify the changes in reflection enhancement as a function of temperature. Overall, the increase in enhancement is 0.08 dB/C for nitrocellulose.

EXAMPLE 5

This example illustrates the temperature-dependent targeting of plasma-rich plasma clots on nitrocellulose membranes using microemulsion ultrasound contrast agent bound to the target with avidin-biotin conjugation.

Biotinylated 1H10 Antibody was prepared using the EZ-LINK Sulfo-NHS-LC-Biotinylation Kit. Briefly, 2 to 10 mg of antibody in 1 ml of phosphate buffered saline is combined with Sulfo-NHS-LC-Biotin in distilled water to afford a 12 to 20-fold molar excess of reagent to antibody. The solution was then incubated at room temperature for 30 minutes. Biotinylated antibody was separated from reagents using a 10 ml desalting column. Equilibration and elution was performed with phosphate buffered saline. Fractions of eluate were collected and UV absorbance measured at 280 nm with a spectrophotometer. Fractions containing antibody were stored at 4°C until use.

Plasma clots were produced on nitrocellulose membrane surfaces by combining human citrated plasma (375 µl) and 100-mM calcium chloride (25µl) with three units of thrombin in a plastic mold placed on the membranes. The plasma was allowed to coagulate slowly at ambient temperature and then transferred to PBS until exposure to the control or targeted contrast system.

Clots (n=9) were incubated with the biotinylated 1H10 antibody overnight in PBS in a 4° C cold room under gentle agitation. The clots were then rinsed and exposed to100µg of avidin under gentle agitation for one hour at room temperature. The clots were then re-rinsed and ready for exposure to contrast agent as described in Example 4. Control clots (n=7), not pretargeted with biotinylated antibody or avidin, were exposed in identical manner to the biotinylated contrast agent.

Measurement of acoustic reflectivity was performed as in Example 4. The inherently poor echogenicity of the native human plasma clots is shown in the left portion of the Figure 4. The figure illustrates two images of the integrated reflected power of a plasma clot before and after delivery of the contrast agent, with the grayscale representing a logarithmic depiction of the reflection enhancement. Darker areas on the right picture represent areas where the enhancement of the clot is increased by the presence of site-targeted ultrasonic contrast agent.

The same plasma clot depicted in Figure 4 is also shown in Figure 5 where the grayscale now represents the change in enhancement brought about by increases in temperature in 5° C increments from the 27° C baseline. As in the case of targeted nitrocellulose, targeted human plasma clots show substantial increase in echogenicity

with temperature while the control clots (unexposed to contrast agent) show little change over this temperature range. Figure 6 shows the results of the reflection enhancement for the 100 brightest sites on the clots averaged over the usable bandwidth of the transducer (17 to 34 MHz). A linear fit to this plot of reflection enhancement vs. temperature yields a correlation coefficient of R=0.99 and a slope of 0.21 dB/C.

In comparison, the control nitrocellulose and control human plasma clots both show only a small change in reflected power as the temperature increases. The targeted or enhanced substrates both exhibit detectable changes in enhancement with as little as a 5° C change in temperature. This differential acoustic reflectivity response can be utilized to segment between contrast targeted and nontargeted surfaces and enhance image presentations through colorization, digital subtraction or similar techniques. The increased sensitivity of targeted tissue detection as well as capability for improved presentation to and recognition by clinical readers could markedly improve the recognition of pathologies that might have otherwise have been unappreciated.

EXAMPLE 6

This example illustrates the lack of hystersis of temperature-dependent change in acoustic reflectivity of avidin-biotin targeted fluorocarbon emulsion bound to human plasma clot.

To test for the reversibility of the temperature effect, two human plasma clot samples were heated and then cooled and the reflection enhancement recorded for each temperature point using techniques described in example 3. The hysteresis curves are plotted in Figure 7. Within the error bars of the measurements, the magnitude of enhancement was identical at each temperature, independent of the direction of temperature change. Given the boiling point of the selected fluorocarbon, i.e. perfluorooctane (105° C), and the heat-dissipating effect of the 37° C water bath in which the samples were insonified, these results confirm that enhancement or diminishment of acoustic reflectivity of targeted tissues results from temperature-

dependent changes in acoustic impedence of the bound emulsion particles and not a phase-transition of the fluorocarbon to a gaseous state.

EXAMPLE 7

This example illustrates methodology and instrumentation that can be used in applying the present invention to ultrasound imaging in conjunction with hyperthermia therapy in specifically detecting, localizing and defining the morphology of the tumor burden and in monitoring the hyperthermia.

Hyperthermia in various forms is now used in conjunction with radiation oncology as an augmentive treatment for various cancers, particularly those less than 8 cm in depth. One device currently used for this application is the Sonotherm® 1000 produced by Labthermics Technologies, Champaign, IL. Optimal tissue hyperthermia depends on heating the tumor with minimum treatment of surrounding normal tissues. SONOTHERM 1000 has the capability to segment a specified tissue volume into small cubic treatment voxels. By adjusting the frequency and intensity of different elements of the ultrasound array, "optimal" heating patterns are achieved.

The keys to all this regimen, especially with regard to difficult to distinguish small tumors, are (1) the precise localization and morphologic delineation of the tumor in a three-dimensional volume space and (2) noninvasive thermometry of the tissue heating process to ensure tumor kill and sparing of normal collateral tissues.

The temperature-dependent ultrasound contrast agents of the present invention will greatly enhance the high-resolution detection, localization and mapping of tumors in two-dimensional or three-dimensional space, particularly when the cancer is small or the background in inherently acoustically reflective. This is achieved through the differential ultrasonic response of nanoparticle-targeted and surrounding normal tissues. In addition, the temperature-dependent changes in acoustic backscatter could be used an internal thermometry, assuring that targeted tissues are heated to appropriate levels while of other tissue heating is minimized. This high resolution, noninvasive thermometry may be constantly displayed in real-time using a scaled color map to allow the operator to monitor tissue temperatures and manually adjust

the hyperthermetry protocol. Alternatively, ultrasonic beam adjustments may be automatically implemented "on-the-fly" by the hyperthermia machine through self-monitoring algorithms.

Pathologies, such as tumors, are often difficult to differentiate from normal tissues with routine ultrasound and benefit from specific contrast enhancement. Unfortunately, the high acoustic reflectivity of adjacent tissues may often diminish the magnitude of contrast enhancement achieved, even with targeted molecular imaging agents. The opportunity to use temperature dependent, targeted molecular contrast agents further provides a unique mechanism for differentiating normal from pathologic tissues by increasing the acoustic signal from targeted versus normal surrounding tissues. This feature will allow refined segmentation and localization of tumors, particularly small tumors, for biopsy or external radiation or hyperthermic therapy. Improved localization affords more precise focusing of external therapeutic energy sources, maximizing the efficiency of treatment and minimizing colateral damage. In addition, the magnitude of increased acoustic backscatter relative to surrounding tissues provides noninvasive thermometry. The proper level of heating at the target site may be monitored and controlled manually or by self-regulating systems within hyperthermia instrumentation. Technical advantages can be immediately envisioned for the treatment of breast cancer, malignant melanoma, sarcomas, lymphomas, head and neck cancers, and soon more deeply positioned tumors such as colon, cervical, uterine, hepatic, pancreatic, gastric and the like.

For instance, a patient or animal with breast cancer is admitted to the hospital, and an intravenous catheter is placed. Temperature-sensitive acoustic nanoparticles bearing a monoclonal antibody fragment directed against $\alpha_v\beta_3$ integrin on neovascular cells is administered at a dose of between 0.1 and 1.0 ml/kg body weight, preferably 0.25 to 0.5 ml/kg body weight. The agent is allowed to circulate and saturate the neovascular tissue receptors for between 15 minutes and 5 hours, preferably 1 to 2 hours. Baseline ultrasound images are obtained with a standard, commercially available ultrasound imaging device, such as those produced Agilent (Andover, MA), Acuson (Mountainview, CA), ATL (Bothel, WA), GE (Farifield, CT) Toshiba (Tokyo, JP), and similar devices. Sonothermography may be instituted with a

SONOTHERM 1000 Therapy system (Labthermics Technologies, Champaign, IL) or a related device according to the manufactures recommendations for the anticipated location of the suspected mass. The SONOTHERM 1000 is cycled to provide intermittent elevations (lasting less than 10 seconds each) in tissue temperature (between 42.° C and 45°C) in the selected region. The differential changes of acoustic contrast of the tumor vasculature targeted with nanoparticles and the surrounding normal tissue are used to specifically detect, localize and define the morphology of the tumor burden. These results are programmed into the SONOTHERM 1000 to refine the location and distribution for the hyperthermic radiation to be imparted. Throughout the subsequent hyperthermia therapy session, the temperature-dependent changes in acoustic backscatter imparted by the targeted nanoparticles within each region of the target tissue may be used to noninvasively determine deep tumor temperature. This information allows fine and continuous regulation of the hyperthermia protocol to closest possible tolerance, minimizing collateral damage to normal tissues and enhancing the overall safety of the procedure

EXAMPLE 8

This example illustrates methodology and instrumentation that can be used in applying the present invention to catheter-directed invasive hyperthermia.

The targeted temperature-dependent nanoparticles can be used in a catheter-based system as follows. A variety of therapeutic ultrasound catheters have been developed that allow highly focused heat generation for therapy and surgical application. An example of such a device is the multielement array system described by Lee et al. (*IEEE transactions of Biomedical Engineering. 4*:880-90, 1999). This unit has been demonstrated both *in vitro* and *in vivo* to achieve a therapeutic temperature rise (above 5° C) over 92% of a target volume of 30 mm x 30 mm x 35 mm. This and similar devices provide exquisite control of temperature distribution. This hyperthermia catheter system could be coupled with commercially available, intravascular ultrasound transducer technology to provide both fine detailed ultrasonic imaging of heated tissues at frequencies ranging from 10 to 50 MHz. These dual

imaging/therapeutic catheters could be utilized for both endoscopic an intravascular applications.

The catheter for hyperthermia and ultrasound system could be used in treating cancer as in the following example. A patient with suspected pancreatic cancer, would be admitted to the hospital, and an intravenous catheter placed. Temperature-sensitive acoustic nanoparticles bearing a monoclonal antibody fragment directed against $\alpha_v\beta_3$ integrin on neovascular cells is administered at a dose of between 0.1 and 1.0 ml/kg body weight, preferably 0.25 to 0.5 ml/kg. The agent is allowed to circulate and saturate the neovascular tissue receptors for between 15 minutes and 5 hours, preferably 1 to 2 hours. A combinational therapeutic/imaging ultrasonic catheter is advanced and images of the pancreas from a transgastric/transduodenal approach are obtained. The diagnosis, location and extend of the pancreatic tumor is confirmed through a temperature-dependent imaging protocol as previously described. The tumor is insonified to induce localized hyperthermia. The temperature of targeted tissue is monitored continuously by the changes in acoustic backscatter. Incremental temperature differences are color-mapped onto ultrasonic image displays that are repetitively updated and reviewed by the operator. The operator manually or the equipment automatically adjusts intensity or frequency of the ultrasonic beam to optimize tumor destruction and minimize collateral damage to normal tissues.

In view of the above, it will be seen that the several advantages of the invention are achieved and other advantageous results attained.

As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above description be interpreted as illustrative and not in a limiting sense.

All references cited in this specification are hereby incorporated by reference. The discussion of the references herein is intended merely to summarize the assertions made by their authors and no admission is made that any reference constitutes prior art. Applicant reserves the right to challenge the accuracy and pertinency of the cited references.

WHAT IS CLAIMED IS:

1. A method for changing acoustic reflectivity of an ultrasound target, the method comprising (1) administering to the target, a nongaseous acoustic imaging substance which binds to the target and produces a change in acoustic reflectivity with a change in temperature and (2) changing the temperature to produce a measurable change in acoustic reflectivity of the nongaseous acoustic imaging substance bound to the target.

2. The method according to claim 1 wherein the nongaseous acoustic imaging substance comprises an emulsion which contains a liquid fluorocarbon.

3. The method according to claim 5 wherein the fluorocarbon is perfluorooctane.

4. The method according to claim 1 wherein the nongaseous acoustic imaging substance comprises a ligand which binds to the target.

5. The method according to claim 4 wherein the ligand is an antibody, a fragment of an antibody, a polypeptide, a peptidomimetic, a polysaccharide, an aptamer, a lipid, a nucleic acid or a lectin.

6. The method according to claim 5 wherein the nongaseous acoustic imaging substance comprises a ligand conjugated with a biotin agent, an emulsion conjugated with a biotin agent and an avidan agent immobilized to the biotin-conjugated ligand and to the biotin-conjugated emulsion.

7. The method according to claim 5 wherein the nongaseous imaging substance comprises a ligand imobilized to a primer substance which is imobilized to an emulsion.

8. The method according to claim 1 wherein the emulsion further comprises a biologically active agent.

9. The method according to claim 8 wherein the biologically active agent is an antineoplastic agent, a radiopharmaceutical, a hormone, an analgesic agent, a nonsteroidal anti-inflammatory agent, an anesthetic agent, a sedative, a neuromuscular blocker, an antimicrobial agent, an antiparasitic agent, an antiviral agent, an interferon, a nitrate, an acne preparation, an androgenic agent, an antidiabetic agent, an anti-gout agent, an antihistamine, an antitussive agent, a decongestant, an expectorant, an antiulcer agent, a laxative, an anticoagulant, an immunization agent, an anticonvulsant agent, an anti-parkinsonian agent, an estrogenic agent, a thyroid agent, or an iron-containing anti-anemia agent.

10. The method according to claim 1 wherein the nongaseous acoustic imaging substance further comprises a magnetic resonance imaging substance, an electron spin resonance imaging substance, a spectroscopic imaging substance, a positron emission tomography imaging substance, an optical imaging substance, an x-ray imaging substance, a nuclear medicine imaging substance or a combination thereof.

11 The method according to claim 1 wherein the spectroscopic imaging substance comprises a nuclear magnetic resonance spectroscopic imaging substance or a raman spectroscopy imaging substance.

12. The method according to claim 11 wherein the nongaseous imaging agent comprises a paramagnetic or superparamagnetic element, a radioactive nuclide, or a photoactive agent.

13. The method according to claim 1 wherein changing the temperature comprises energizing the bound substance to increase temperature of the bound substance and enhance acoustic reflectivity of the surface.

14. The method according to claim 13 wherein the energizing is performed by generating energy from ultrasound, shortwave, microwave, magnetic radiation, electromagnetic energy or a combination thereof.

15. The method according to claim 1 wherein changing the temperature of the bound substance comprises reducing the temperature of the bound substance to produce a measurable decrease in acoustic reflectivity of the target.

16. The method according to 15 wherein reducing the temperature of the bound substance is performed as part of cryotherapy or heart bypass surgery.

17. The method according to claim 1 wherein changing the temperature comprises changing the temperature of the bound substance by at least 5°C.

18. A method for measuring enhanced acoustic reflectivity of an ultrasound target, the method comprising (1) administering to the target, a nongaseous acoustic imaging substance which binds to the target and produces a change in acoustic reflectivity with a change in temperature and (2) changing the temperature to produce a measurable change in acoustic reflectivity of the nongaseous acoustic imaging substance bound to the target, and (3) detecting change in acoustic reflectivity of the bound substance.

19. The method according to claim 18 wherein detecting comprises (a) measuring reflectivity prior to changing the temperature of the bound substance; (b) measuring reflectivity after changing the temperature of the bound substance; and (c) determining the change in reflectivity after changing the temperature of the bound substance compared to reflectivity prior to changing the temperature of the bound substance.

20. The method according to claim 19 wherein the nongaseous acoustic imaging substance comprises an emulsion which contains a liquid fluorocarbon.

21. The method according to claim 20 wherein the fluorocarbon is perfluorooctane.

22. The method according to claim 19 wherein the nongaseous acoustic imaging substance comprises a ligand which binds to the target.

23. The method according to claim 22 wherein the ligand is an antibody, a fragment of an antibody, a polypeptide, a peptidomimetic, a polysaccharide, an aptamer, a lipid, a nucleic acid or a lectin.

24. The method according to claim 23 wherein the nongaseous acoustic imaging substance comprises a ligand conjugated with a biotin agent, an emulsion conjugated with a biotin agent and an avidan agent immobilized to the biotin-conjugated ligand and to the biotin-conjugated emulsion.

25. The method according to claim 23 wherein the nongaseous imaging substance comprises a ligand immobilized to a primer substance which is immobilized to an emulsion.

26. The method according to claim 19 wherein the emulsion further comprises a biologically active agent.

27. The method according to claim 26 wherein the biologically active agent is an antineoplastic agent, a radiopharmaceutical, a hormone, an analgesic agent, a nonsteroidal anti-inflammatory agent, an anesthetic agent, a sedative, a neuromuscular blocker, an antimicrobial agent, an antiparasitic agent, an antiviral agent, an interferon, a nitrate, an acne preparation, an androgenic agent, an antidiabetic agent, an anti-gout agent, an antihistamine, an antitussive agent, a decongestant, an expectorant, an antiulcer agent, a laxative, an anticoagulant, an immunization agent, an anticonvulsant agent, an anti-parkinsonian agent, an estrogenic agent, a thyroid agent, or an iron-containing anti-anemia agent.

28. The method according to claim 19 wherein the nongaseous acoustic imaging substance further comprises a magnetic resonance imaging substance, an electron spin resonance imaging substance, a spectroscopic imaging substance, a positron emission tomography imaging substance, an optical imaging substance, an x-ray imaging substance, a nuclear medicine imaging substance or a combination thereof.

29. The method according to claim 19 wherein the spectroscopic imaging substance comprises a nuclear magnetic resonance spectroscopic imaging substance or a raman spectroscopy imaging substance.

30. The method according to claim 29 wherein the nongaseous imaging agent comprises a paramagnetic or superparamagnetic element, a radioactive nuclide, or a photoactive agent.

31. The method according to claim 19 wherein changing the temperature comprises energizing the bound substance to increase temperature of the bound substance and enhance acoustic reflectivity of the surface.

32. The method according to claim 31 wherein the energizing is performed by generating energy from ultrasound, shortwave, microwave, magnetic radiation, electromagnetic energy or a combination thereof.

33. The method according to claim 19 wherein changing the temperature of the bound substance comprises reducing the temperature of the bound substance to produce a measurable decrease in acoustic reflectivity of the target.

34. The method according to 33 wherein reducing the temperature of the bound substance is performed as part of cryotherapy or heart bypass surgery.

35. The method according to claim 19 wherein changing the temperature comprises changing the temperature of the bound substance by at least 5°C.

36. A method for monitoring temperature of a tissue in a patient, the method comprising (1) administering to the patient, a nongaseous acoustic imaging substance which binds to the tissue and changes acoustic reflectivity with changes in temperature, (2) detecting acoustic reflectivity of the nongaseous acoustic imaging substance bound to the tissue (3) calculating temperature of the nongaseous acoustic imaging substance bound to the tissue.

37. The method according to claim 36 wherein the method monitors a change in temperature, wherein the method further comprises changing the temperature of the tissue and the nongaseous acoustic imaging substance bound to the tissue, and wherein detecting comprises detecting the change in acoustic reflectivity of the nongaseous acoustic imaging substance bound to the tissue.

38. The method according to claim 36 wherein the nongaseous acoustic imaging substance comprises an emulsion which contains a liquid fluorocarbon.

39. The method according to claim 38 wherein the fluorocarbon is perfluorooctane.

40. The method according to claim 36 wherein the nongaseous acoustic imaging substance comprises a ligand which binds to the tissue.

41. The method according to claim 40 wherein the ligand is an antibody, a fragment of an antibody, a polypeptide, a peptidomimetic, a polysaccharide, an aptamer, a lipid, a nucleic acid or a lectin.

42. The method according to claim 41 wherein the nongaseous acoustic imaging substance comprises a ligand conjugated with a biotin agent, an emulsion conjugated with a biotin agent and an avidan agent immobilized to the biotin-conjugated ligand and to the biotin-conjugated emulsion.

43. The method according to claim 41 wherein the nongaseous imaging substance comprises a ligand immobilized to a primer substance which is immobilized to an emulsion.

44. The method according to claim 36 wherein the emulsion further comprises a biologically active agent.

45. The method according to claim 44 wherein the biologically active agent is an antineoplastic agent, a radiopharmaceutical, a hormone, an analgesic agent, a nonsteroidal anti-inflammatory agent, an anesthetic agent, a sedative, a neuromuscular blocker, an antimicrobial agent, an antiparasitic agent, an antiviral agent, an interferon, a nitrate, an acne preparation, an androgenic agent, an antidiabetic agent, an anti-gout agent, an antihistamine, an antitussive agent, a decongestant, an expectorant, an antiulcer agent, a laxative, an anticoagulant, an immunization agent, an anticonvulsant agent, an anti-parkinsonian agent, an estrogenic agent, a thyroid agent, or an iron-containing anti-anemia agent.

46. The method according to claim 36 wherein the nongaseous acoustic imaging substance further comprises a magnetic resonance imaging substance, an electron spin resonance imaging substance, a spectroscopic imaging substance, a positron emission tomography imaging substance, an optical imaging substance, an x-ray imaging substance, a nuclear medicine imaging substance or a combination thereof.

47. The method according to claim 46 wherein the spectroscopic imaging substance comprises a nuclear magnetic resonance spectroscopic imaging substance or a raman spectroscopy imaging substance.

48. The method according to claim 47 wherein the nongaseous acoustis imaging agent comprises a paramagnetic or superparamagnetic element, a radioactive nuclide, or a photoactive agent.

49. The method according to claim 36 wherein changing the temperature comprises energizing the bound substance to increase temperature of the bound substance and enhance acoustic reflectivity of the surface.

50. The method according to claim 49 wherein the energizing is performed by generating energy from ultrasound, shortwave, microwave, magnetic radiation, electromagnetic energy or a combination thereof.

51. The method according to claim 36 wherein changing the temperature of the bound substance comprises reducing the temperature of the bound substance to produce a measurable decrease in acoustic reflectivity of the target.

52. The method according to 51 wherein reducing the temperature of the bound substance is performed as part of cryotherapy or heart bypass surgery.

53. The method according to claim 36 wherein changing the temperature comprises changing the temperature of the bound substance by at least 5°C.

54. A device for measuring changes in temperature of a target having a temperature-sensitive acoustic imaging substance bound thereto, the device comprising a component configured to change the temperature of the acoustic imaging substance, an ultrasound source configured to transmit acoustic energy to the target, an ultrasound detecting component configured to measure acoustic reflectivity of the surface and a comparator which determines acoustic reflectivity of the target upon changing temperature relative to acoustic reflectivity of the target in absence of changing temperature.

55. The device according to claim 54 wherein the comparator determines difference in acoustic reflectivity of the target prior to and after changing temperature of the acoustic imaging substance bound to the target.

56. The device according to claim 55 wherein the comparator determines the difference in acoustic reflectivity of the target upon changing temperature of the acoustic imaging substance bound to the target, compared to acoustic reflectivity of the target after the changed temperature of the acoustic imaging substance bound to the target is diminished.

57. The device according to claim 54 wherein the ultrasound source and the ultrasound imaging component comprise at least one ultrasonic transducer.

58. The device according to claim 54 wherein the component configured to change the temperature of the acoustic imaging substance comprises an energy source.

59. The device according to claim 58 wherein the eneregy source produces ultrasound, shortwave, microwave, magnetic radiation, electromagnetic energy or a combination thereof.

60. The device according to claim 59 wherein the energy source is an ultrasound energy source having an intensity of greater than 0.1 W/cm^2 and less than 2000 W/cm^2.

61. The device according to claim 54 wherein the component configured to change the temperature of the acoustic imaging substance is an energy absorber.

62. The device according to claim 61 wherein the energy absorber comprises a cryogenic probe.

63. The device according to claim 54 wherein the component confugured to change the temperature of the acoustic imaging substance is configured to change the temperature of the bound substance by at least 5°C.

64. The device according to claim 54 wherein the comparator is configured to produce an image comprising the difference in acoustic reflectivity of the surface prior to and after increasing temperature of the acoustic imaging substance.

65. The device according to claim 64 wherein the comparator is configured to produce a colorized image comprising the difference in acoustic reflectivity of the surface prior to and after increasing temperature of the acoustic imaging substance.

66. The device according to claim 54 further comprising a component configured to perform magnetic resonance imaging, electron spin resonance imaging , spectroscopic imaging, positron emission tomography imaging, optical imaging, x-ray imaging nuclear medicine imaging or a combination thereof.

67. The method according to claim 66 wherein the spectroscopic imaging comprises nuclear magnetic resonance spectroscopic imaging or a raman spectroscopy imaging.

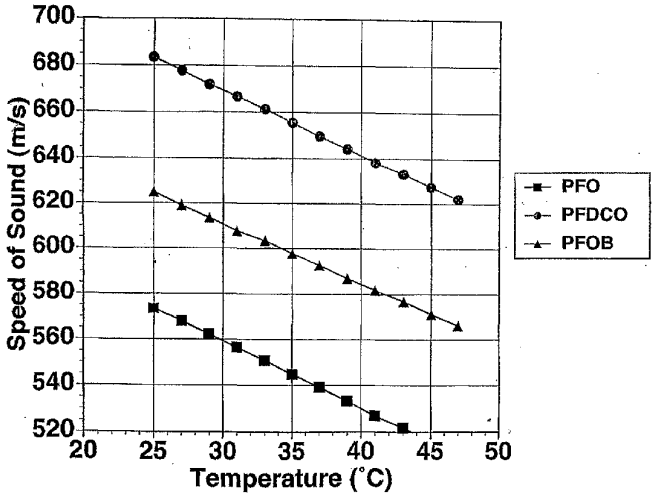

700
680
660
640
620
600
580
560
540
520
20
25
30
35
40
45
50
Speed of Sound (m/s)
Temperature (˚C)
PFO
PFDCO
PFOB

Figure 1

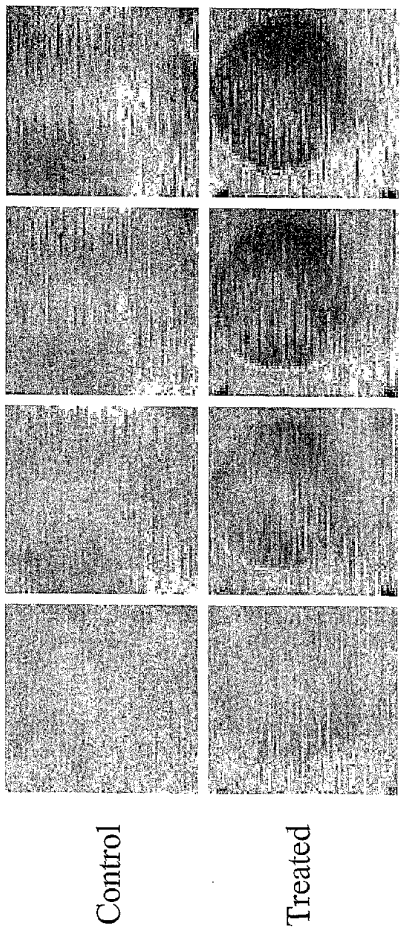


Figure 2

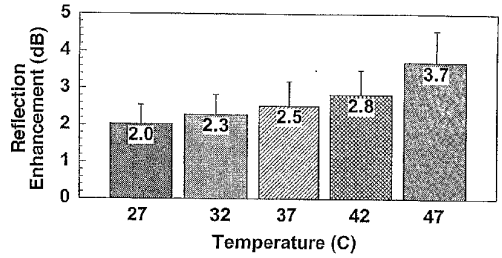
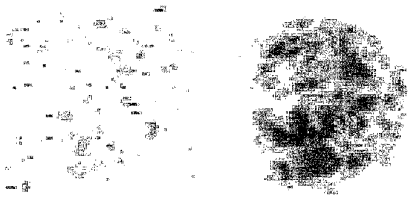

Reflection Enhancement (dB)
5
4
3
2
1
0
2.0
2.3
2.5
2.8
3.7
27
32
37
42
47
Temperature (C)

Figure 3



Before After

Figure 4

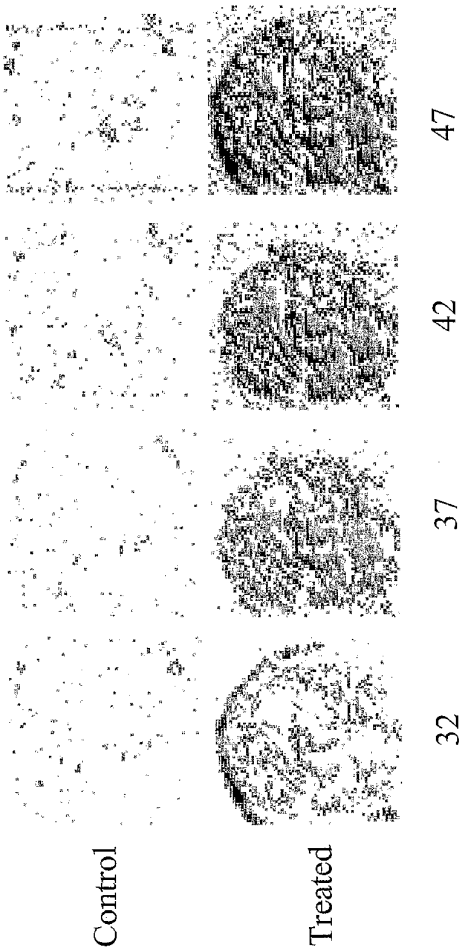


Figure 5

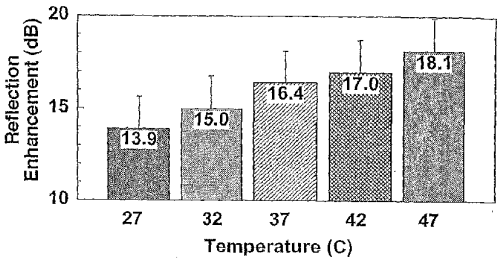

Reflection Enhancement (dB)
20
15
10
13.9
15.0
16.4
17.0
18.1
27
32
37
42
47
Temperature (C)

Figure 6

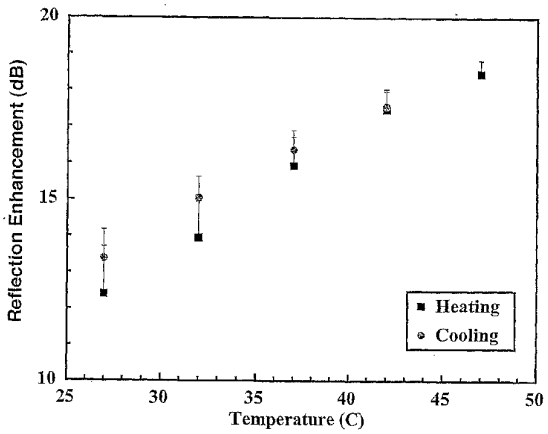

20
15
10
Reflection Enhancement (dB)
25
30
35
40
45
50
Temperature (C)
Heating
Cooling

Figure 7

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(54) Title: ENHANCED ULTRASOUND DETECTION WITH TEMPERATURE-DEPENDENT CONTRAST AGENTS

(57) Abstract: Methods and devices for enhanced ultrasound detection based upon changing temperature and ultrasound reflectivity of a temperature-dependent contrast agent bound to an ultrasound target are disclosed. The methods and devices can be used for enhanced imaging alone or in conjunction with drug delivery, with therapeutic approaches such as hyperthermia or cryotherapy or with other imaging modalities.

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(54) Title: ENHANCED ULTRASOUND DETECTION WITH TEMPERATURE DEPENDENT CONTRAST AGENTS

(57) Abstract: Methods and devices for enhanced ultrasound detection based upon changing temperature and ultrasound reflectivity of a temperature-dependent contrast agent bound to an ultrasound target are disclosed. The methods and devices can be used for enhanced imaging alone or in conjunction with drug delivery, with therapeutic approaches such as hyperthermia or cryotherapy or with other imaging modalities.

ENHANCED ULTRASOUND DETECTION WITH TEMPERATURE-DEPENDENT CONTRAST AGENTS

BACKGROUND OF THE INVENTION

(1) Field Of The Invention

This invention relates generally to ultrasound detection and imaging and, more particularly, to novel compositions, methods and devices for detecting a change in ultrasound reflectivity based upon changing the temperature of a temperature-dependent contrast agent bound to the target.

(2) Description Of The Related Art

Molecular imaging can enhance the utility of traditional clinical imaging by allowing specific detection of molecular markers in tissues using site-targeted contrast agents (Weissleder, *Radiology 212*:609-614, 1999). Three approaches to site-targeted ultrasonic agents have been reported and these are based upon the use of liposomes (Alkan-Onyuksel et al., *J. Pharm. Sci 85*:486-490, 1996; Demos et al., *J. Pharm. Sci. 86*:167-171, 1997; Demos et al., *J. Am. Col. Cardiol. 33*:867-875, 1999), the use of microbubbles (Mattrey et al, *Am. J. Cardiol. 54*:206-210, 1984; Unger et al., *Am. J. Cardiol. 81*:58G-61G, 1998; Villanueva et al, *Circulation 98*:1-5, 1998; Klibanov et al, *Acad. Radiol. 5*S243-S246, 1998) or the use of nano-emulsions (Lanza et al, *Circulation 94*:3334-3340, 1996; Lanza et al, *J. Acoust. Soc. Am. 104*:3665-3672, 1998; Lanza et al, *Ultrasound Med. Biol. 23*: 863-870, 1997). Liposomes are spherical bimembrane vesicles produced spontaneously by phospholipids in water. Multilamellar lipid bilayers produced through a dehydration-rehydration process can form internal vesicles within a liposome and lead to increased acoustic reflectance (Alkan-Onyuksel et al., 1996 *supra*; Demos et al., 1997, *supra*; Demos et al., 1999, *supra*). In the second approach, microbubbles have been proposed for site-targeted modalities in addition to their perfusion applications. Microbubbles have been targeted towards thrombi (Unger et al., 1998m supra; Lanza et al., *Ultrasound. Med. Biol. 23*: 863-870, 1997), avidin-coated petri dish (Klibanov et al, 1998, *supra*) and activated endothelial cells (Villanueva et al, 1998, *supra*). Other investigators have examined the interaction of thrombus with site targeted agents. In particular, Unger et

al. has observed successful binding of MRX-408, a bubble-based contrast agent, both *in vitro* and *in vivo* (Unger et al., 1998, *supra*).

The site-targeted nano-emulsions are nongaseous acoustic contrast agents made up of lipid-encapsulated liquid perfluorocarbon nanoparticules (see Lanza et al., U.S. Patent Nos. 5,690,907,; 5,780,010; and 5,958,371). The nanoparticles are approximately 250 nm in diameter. Perfluorocarbon nanopatriculate emulsions have been shown to provide substantial acoustic contrast when targeted towards *in vitro* and *in vivo* thrombi preparations (Lanza et al., 1998, *supra*; Lanza et al., 1997, *supra*).

One of the challenges confronting the use of site-targeted contrast agents is the sensitive detection and differentiation of the particles from the surrounding soft tissue. Detection of pathological changes on or near vascular surfaces may be compromised because the targeted substrate itself is echogenic or the signal from that surface may be somewhat view or angle dependent. Imaging techniques have been developed in attempts to solve this issue. Second harmonic or harmonic and power harmonic Doppler imaging has been used to allow differentiation of microbubbles in circulation from tissue (see Burns et al. *Clinical Radiol. 51*:50-55, 1996; Kasprzak et al, *Am. J. Cardiol. 83*:211-217, 1999; Senior et al, *Am. Heart J. 139*:245-251, 2000; Spencer et al, *J. Am. Soc. Echo. 13*:131-138, 2000). However, soft tissue also exhibits a second harmonic backscattered signal. Furthermore, the contrast agent may manifest velocities too slow for the sensitivity of Doppler techniques. Unlike the resonance phenomenon responsible for enhanced backscatter cross section in microbubbles, the mechanism for increased reflection enhancement from the site-targeted nanoparticle emulsions has been reported to be due to acoustic impedance mismatch at the surface where the particles bind (Lanza et al., 1998, *supra*). Thus, although site-targeted acoustic contrast agents and, in particular, the nano-emulsion contrast agents have been used as contrast agents, the development of approaches that produce a greater degree of contrast could potentially provide further sensitivity for ultrasound molecular imaging systems.

Perfluorocarbon liquids are known to transmit ultrasound at low velocities (Lagemann et al. *J. Am. Chem Soc. 70*: 2994-2996, 1948; Gupta, *Acustica 42*:273-277, 1979). The low ultrasound velocities through these substances have been shown to be

temperature dependent in that the ultrasound velocity is decreased in a linear manner with increasing temperature (Narayana et al., *Acoustics Letters 9*:137-143, 1986). This observation was reported to be potentially applicable to the development of acoustic lenses (*Id.*). Nevertheless, the temperature-dependence of ultrasound velocity in perfluorocarbon liquids has not, heretofore, been suggested to have any applicability in ultrasound imaging systems.

Ultrasound energy has been applied in site-targeted contrast agents in ultrasound imaging methods as noted above. Much of this earlier work was directed to molecular imaging so that only low level ultrasound energy was used and no change in temperature of the targeted surface was reported to occur. In microbubble ultrasound imaging systems, sufficient energy has been applied to a liquid precursor substance to form gaseous microbubbles. (Lohrmann et al., U.S. Patent No. 5,536,489; Unger, U.S. Patent No. 5,542,935). One suggested approach has been to apply the energy to produce the phase shift *in vivo*. In such approaches, temperature changes would serve to convert the gaseous precursor to the gaseous microbubbles and none of these earlier studies disclosed or suggested changing temperature of an ultrasound contrast agent which remains in the liquid state or using the change in temperature of a nongaseous contrast agent as a basis for enhancing ultrasound detection.

Thus, there remains a continuing need for developing approaches that produce an enhanced degree of contrast and provide further sensitivity for ultrasound molecular imaging systems.

BRIEF SUMMARY OF THE INVENTION

Accordingly, the inventors herein have succeeded in discovering that changing the temperature of nanoparticles which contain a nongaseous fluorocarbon liquid and which are bound to a target, produces a detectable change in acoustic reflectivity of the target. Non-targeted regions which are adjacent to the target, but are not bound by the nanoparticles, show little or no detectable change in acoustic reflectivity. As a result, the temperature-dependent change in acoustic reflectivity of site-targeted

nanoparticles provides a sensitive measurement of ultrasound reflectivity and provides enhanced contrast imaging.

Thus, in one embodiment, the present invention is directed to a method for changing acoustic reflectivity of an ultrasound target. The method comprises (1) administering to the target, a nongaseous acoustic imaging substance which binds to the target and produces a change in acoustic reflectivity with a change in temperature and (2) changing the temperature to produce a measurable change in acoustic reflectivity of the nongaseous acoustic imaging substance bound to the target. The nongaseous acoustic imaging substance, preferably, comprises a nanoparticle emulsion which contains a liquid fluorocarbon. The nongaseous acoustic imaging substance, preferably, comprises a ligand which binds to the target.

In another embodiment, the present invention comprises a method for measuring enhanced acoustic reflectivity of an ultrasound target. The method comprises (1) administering to the target, a nongaseous acoustic imaging substance which binds to the target and produces a change in acoustic reflectivity with a change in temperature and (2) changing the temperature to produce a measurable change in acoustic reflectivity of the nongaseous acoustic imaging substance bound to the target, and (3) detecting change in acoustic reflectivity of the bound substance. Detecting the change in acoustic reflectivity, preferably, comprises (a) measuring reflectivity prior to changing the temperature of the bound substance; (b) measuring reflectivity after changing the temperature of the bound substance; and (c) determining the change in reflectivity after changing the temperature of the bound substance compared to reflectivity prior to changing the temperature of the bound substance.

Another embodiment of the present invention involves a method for monitoring temperature of a tissue in a patient. The method comprises (1) administering to the patient, a nongaseous acoustic imaging substance which binds to the tissue and changes acoustic reflectivity with changes in temperature, (2) detecting acoustic reflectivity of the nongaseous acoustic imaging substance bound to the tissue (3) calculating temperature of the nongaseous acoustic imaging substance bound to the tissue. Preferably, the method monitors a change in temperature and the method further comprises changing the temperature of the tissue and the nongaseous acoustic

imaging substance bound to the tissue. Detecting acoustic reflectivity comprises detecting the change in acoustic reflectivity of the nongaseous acoustic imaging substance bound to the tissue.

In one aspect of the present invention, the change in temperature can be produced by energizing the bound nongaseous nanoparticles to increase temperature of the bound substance and enhance acoustic reflectivity of the target. The nanoparticles can be energized by ultrasound, shortwave, microwave, magnetic radiation, electromagnetic energy or a combination thereof.

In another aspect of the present invention, the temperature of the bound nanoparticles can be decreased to produce a measurable decrease in acoustic reflectivity of the target.

The methods of the present invention can be used in conjunction with administration with a biologically active agent which is incorporated into the nanoparticle. In addition, other imaging techniques can be used with the acoustic imaging upon incorporating into the nanoparticle one or more imaging agents suitable for use in such other imaging techniques such as, for example, magnetic resonance imaging, electron spin resonance imaging, spectroscopic imaging, positron emission tomography imaging, optical imaging, x-ray imaging, nuclear medicine imaging or a combination thereof.

In another embodiment, the present invention comprises a device for measuring changes in temperature of a target to which a temperature-sensitive acoustic imaging substance is bound. The device comprises a component configured to change the temperature of the acoustic imaging substance, an ultrasound source configured to transmit acoustic energy to the target, an ultrasound detecting component configured to measure acoustic reflectivity of the surface and a comparator which determines acoustic reflectivity of the target upon changing temperature relative to acoustic reflectivity of the target in absence of changing temperature. In one aspect, the comparator determines difference in acoustic reflectivity of the target prior to and after changing temperature of the acoustic imaging substance bound to the target. In another aspect, the comparator determines the difference in acoustic reflectivity of the target upon changing temperature of the acoustic imaging substance

bound to the target, compared to acoustic reflectivity of the target after the changed temperature of the acoustic imaging substance bound to the target is diminished. The temperature changing component can comprise an energy source which increases the temperature of acoustic imaging substance bound to the target or an energy absorbing component which decreases the temperature of the acoustic imaging substance bound to the target.

The device can also comprise a component which performs at least one other imaging technique such as, for example, magnetic resonance imaging, electron spin resonance imaging , spectroscopic imaging, positron emission tomography imaging, optical imaging, x-ray imaging nuclear medicine imaging or a combination thereof.

Among the several advantages achieved by the present invention, therefore, may be noted the provision of methods for enhancing acoustic reflectivity of a target; the provision of methods for distinguishing a target tissue from surrounding tissue which is acoustically reflective; the provision of methods for detecting and monitoring temperature of a tissue; the provision of methods for detecting temperature changes in a tissue such as during a therapeutic treatment involving a change in temperature; and the provision of devices for performing such methods.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the decrease in the speed of sound transmission with increasing temperatures for the perfluorocarbons, perfluorooctane (PFO), perfluorodichlorooctane (PFDCO) and perfluorooctylbromide (PFOB).

Figure 2 illustrates the differential ultrasound images obtained from nitrocellulose membranes showing the increase in reflectivity over that measured at 27° C for temperatures of 32° C, 37° C, 42° C, and 47° C in which darker grays indicate greater enhancement of reflectivity.

Figure 3 illustrates the relative change of ultrasonic reflected power as a function of temperature between targeted and control nitrocellulose membranes.

Figure 4 illustrates the ultrasonic reflection from a human fibrin clot before (left panel) and after (right panel) targeting with contrast agent wherein darker grays represent larger reflection..

Figure 5 illustrates the differential ultrasound images obtained from human plasma clots on nitrocellulose membranes showing the increase in reflectivity over that measured at 27° C for temperatures of 32° C, 37° C, 42° C, and 47° C in which darker grays indicate greater enhancement of reflectivity.

Figure 6 illustrates the relative change of ultrasonic reflected power as a function of temperature between targeted and control human plasma clots on nitrocellulose membranes.

Figure 7 illustrates the lack of hystersis of the reflection enhancement from human plasma clot plotted as a function of temperature when heated (circles) and then cooled (squares).

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, it has been discovered that the detection of acoustic reflectivity of an ultrasound target can be enhanced by changing the temperature of a nongaseous acoustic imaging substance which is bound to the target and which exhibits a measurable change in acoustic reflectivity with a change in temperature.

The acoustic imaging substance is, preferably, a nanoparticle emulsion as has been described earlier (see U.S. Patent Nos. 5,780,010, 5,958,371 and 5,989,520). The nanoparticle emulsions of the present invention are comprised of at least two immiscible liquids which are intimately dispersed, preferably, a hydrophobic material such as an oil, dispersed in water. The emulsions are in the form of droplets or nanoparticles having a diameter which typically exceeds 0.1 μ. Additives such as surface-active agents or finely-divided solids can be incorporated into the emulsion nanoparticles to increase their stability.

The oil phase of the oil-in-water emulsion comprises, preferably, 5 to 50% and, more preferably 20 to 40% by weight of the emulsion. The oil or hydrophobic

constituent exhibits an acoustic impedance that varies with changes (i.e. positively or negatively) in temperature, preferably, a fluorochemical liquid. These include straight, branched chain and cyclic perfluorocarbons, straight , branched chain and cyclic perfluoro tertiary amines, straight , branched chain and cyclic perfluoro ethers and thioethers, chlorofluorocarbons and polymeric perfluoro ethers and the like. Although up to 50% hydrogen-substituted compounds can be used, perhalo compounds are preferred. Most preferred are perfluorinated compounds. Any fluorochemical liquid, i.e. a substance which is a liquid at or above body temperature (e.g. 37°C) at atmospheric pressure, can be used to prepare a fluorochemical emulsion of the present invention. However, for many purposes emulsions fluorochemicals with longer extended stability are preferred. In order to obtain such emulsions, fluorochemical liquids with boiling points above 50°C are preferred, and most preferred are fluorochemical liquids with boiling points above about 80°C. The guiding determinant should be that the oil, e.g. a fluorochemical, should be expected to remain in a liquid phase (less than10% gas conversion) under the intended conditions of themal induction and imaging.

Reference to the term "nongaseous" or "liquid" in the context of the nanoparticle emulsions of the present invention is intended to mean that less than 10% of the interior volume of the nanoparticles is in a gas phase per total volume of the nanoparticles (i.e. v/v), more preferably, no more than about 8% (v/v), more preferably no more than about 5% (v/v), and most preferably, no more than than 2% (v/v) or less. The term "about" as used herein is intended to encompass a range of values 10% above and below a stated value such that, for example, about 8% is intended to encompass the range of values from 7.2% to 8.8%.

The nanoparticle emulsions of the present invention are, preferably, lipid encapsulated. In a specific example, the lipid encapsulated particles may be constituted by a perfluorocarbon emulsion, the emulsion particles having an outer coating of a derivatized natural or synthetic phospholipid, a fatty acid, cholesterol, lipid, sphingomyelin, tocopherol, glucolipid, sterylamine, cardiolipin, a lipid with ether or ester linked fatty acids or a polymerized lipid.

Fluorocarbon emulsions and, in particular, perfluorocarbon emulsions are well suited for biomedical applications and for use in the practice of the present invention. The perfluorocarbon emulsions are known to be stable, biologically inert and readily metabolized, primarily by trans-pulmonic alveolae evaporation. Further, their small particle size easily accommodates transpulmonic passage and their circulatory half-life (4-8 hours) advantageously exceeds that of other agents. Also, perfluorocarbons have been used to date in a wide variety of biomedical applications, including use as artificial blood substitutes. For use in the present invention, various fluorocarbon emulsions may be employed including those in which the fluorocarbon is a fluorocarbon-hydrocarbon, a perfluoroalkylated ether, polyether or crown ether. Useful perfluorocarbon emulsions are disclosed in U.S. Pat. Nos. 4,927,623, 5,077,036, 5,114,703, 5,171,755, 5,304,325, 5,350,571, 5,393,524, and 5,403,575 and include those in which the perfluorocarbon compound is perfluorotributylamine, perfluorodecalin, perfluorooctylbromide, perfluorodichlorooctane, perfluorodecane, perfluorotripropylamine, perfluorotrimethylcyclohexane or other perfluorocarbon compounds. Further, mixtures of such perfluorocarbon compounds may be incorporated in the emulsions utilized in the practice of the invention.

As a specific example of a perfluorocarbon emulsion useful in the invention may be mentioned a perfluorodichlorooctane emulsion wherein the lipid coating thereof contains between approximately 50 to 99.5 mole percent lecithin, preferably approximately 55 to 70 to mole percent lecithin, 0 to 50 mole percent cholesterol, preferably approximately 25 to 45 mole percent cholesterol and approximately 0.5 to 10 mole percent biotinylated phosphatidylethanolamine, preferably approximately 1 to 5 mole percent biotinylated phosphatidylethanolamine. Other phospholipids such as phosphatidylserine may be biotinylated, fatty acyl groups such as stearylamine may be conjugated to biotin, or cholesterol or other fat soluble chemicals may be biotinylated and incorporated in the lipid coating for the lipid encapsulated particles. The preparation of an exemplary biotinylated perfluorocarbon for use in the practice of the invention is described hereinafter in accordance with known procedures.

When the lipid encapsulated particles are constituted by a liposome rather than an emulsion, such a liposome may be prepared as generally described in the literature

(see, for example, Kimelberg et al., *CRC Crit. Rev. Toxicol.* 6:25, 1978; Yatvin et al., *Medical Physics* 9:149, 1982). Liposomes are known to the art and generally comprise lipid materials including lecithin and sterols, egg phosphatidyl choline, egg phosphatidic acid, cholesterol and alpha-tocopherol.

Emulsifying agents, for example surfactants, may be used to facilitate the formation of emulsions and increase their stability. Typically, aqueous phase surfactants have been used to facilitate the formation of emulsions of fluorochemical liquids. A surfactant is any substance that contains both hydrophilic and a hydrophobic portions. When added to water or solvents, a surfactant reduces the surface tension. Preferred surfactants are phospholipids and cholesterol.

Any or a variety of lipid surfactants may be incorporated into the lipid monolayer preferably natural or synthetic phospholipids, but also fatty acids, cholesterols, lysolipids, sphingomyelins, tocopherols, glucolipids, stearylamines, cardiolipins, plasmalogens, a lipid with ether or ester linked fatty acids, polymerized lipids, and lipid conjugated polyethylene glycol. Other known surfactant additives such as PLURONIC F-68, HAMPOSYL L30 (W.R. Grace Co., Nashua, N.H.), sodium dodecyl sulfate, Aerosol 413 (American Cyanamid Co., Wayne, N.J.), Aerosol 200 (American Cyanamid Co.), LIPOPROTEOL LCO (Rhodia Inc., Mammoth, N.J.), STANDAPOL SH 135 (Henkel Corp., Teaneck, N.J.), FIZUL 10-127 (Finetex Inc., Elmwood Park, N.J.), and CYCLOPOL SBFA 30 (Cyclo Chemicals Corp., Miami, Fla.); amphoterics, such as those sold with the trade names: Deriphat.TM. 170 (Henkel Corp.), LONZAINE JS (Lonza, Inc.), NIRANOL C2N-SF (Miranol Chemical Co., Inc., Dayton, N.J.), AMPHOTERGE W2 (Lonza, Inc.), and AMPHOTERGE 2WAS (Lonza, Inc.); non-ionics, such as those sold with the trade names: PLURONIC F-68 (BASF Wyandotte, Wyandotte, Mich.), PLURONIC F-127 (BASF Wyandotte), BRIJ 35 (ICI Americas; Wilmington, Del.), TRITON X-100 (Rohm and Haas Co., Philadelphia, Pa.), BRIJ 52 (ICI Americas), SPAN 20 (ICI Americas), GENEROL 122 ES (Henkel Corp.), TRITON N-42 (Rohm and Haas Co.,), Triton.TM. N-101 (Rohm and Haas Co.,), TRITON X-405 (Rohm and Haas Co.,), TWEEN 80 (ICI Americas), TWEEN 85 (ICI Americas), and BRIJ 56 (ICI Americas) and the like, may be used

alone or in combination in amounts of 0.10 to 5.0% by weight to assist in stabilizing the emulsions.

Fluorinated surfactants which are soluble in the fluorochemical liquid to be emulsified can also be used. Suitable fluorochemical surfactants include perfluorinated alkanoic acids such as perfluorohexanoic and perfluorooctanoic acids and amidoamine derivatives. These surfactants are generally used in amounts of 0.01 to 5.0% by weight, and preferably in amounts of 0.1 to 1.0%. Other suitable fluorochemical surfactants include perfluorinated alcohol phosphate esters and their salts; perfluorinated sulfonamide alcohol phosphate esters and their salts; perfluorinated alkyl sulfonamide alkylene quaternary ammonium salts; N,N-(carboxyl-substituted lower alkyl) perfluorinated alkyl sulfonamides; and mixtures thereof. As used herein, the term "perfluorinated" means that the surfactant contains at least one perfluorinated alkyl group.

Suitable perfluorinated alcohol phosphate esters include the free acids of the diethanolamine salts of mono- and bis(1H,1H,2H,2H-perfluoroalkyl)phosphates. The phosphate salts, available under the tradename ZONYL RP (E.I. Dupont de Nemours and Co., Wilmington, Del.), are converted to the corresponding free acids by known methods. Suitable perfluorinated sulfonamide alcohol phosphate esters are described in U.S. Pat. No. 3,094,547. Suitable perfluorinated sulfonamide alcohol phosphate esters and salts of these include perfluoro-n-octyl-N-ethylsulfonamidoethyl phosphate, bis(perfluoro-n-octyl-N-ethylsulfonamidoethyl) phosphate, the ammonium salt of bis(perfluoro-n-octyl-N-ethylsulfonamidoethyl)phosphate,bis(perfluorodecyl-N-ethylsulfonamidoethyl)-phosphate and bis(perfluorohexyl-N ethylsulfonamidoethyl)-phosphate. The preferred formulations use phosphatidylcholine, derivatized-phosphatidylethanolamine and cholesterol as the aqueous surfactant.

Lipid encapsulated emulsions may be formulated with cationic lipids in the surfactant layer that facilitate the adhesion of nucleic acid material to particle surfaces. Cationic lipids may include but are not limited to 1,2-Diacyl-3-Trimethylammonium-Propane (TAP), 1,2-Diacyl-3-Dimethylammonium-Propane (DAP), DC-Cholesterol (DC-Chol), Dimethyldioctadecylammonium Bromide (DDAB), 1,2-Diacyl-sn-Glycero-3-Ethylphosphocholine DOTMA, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-

trimethylammoium chloride; DOTAP, 1,2-dioleoyloxy-3-(trimethylammonio)propane; and DOTB,1,2-dioleoyl-3-(4'-trimethyl-ammonio)butanoyl-sn-glycerol may be used. In general the molar ratio of cationic lipid to non-cationic lipid in the lipid surfactant monolayer may be, for example, 1:1000 to 2:1, preferably, between 2:1 to 1:10, more preferably in the range between 1:1 to 1:2.5 and most preferably 1:1 (ratio of mole amount cationic lipid to mole amount non-cationic lipid, e.g., DPPC). A wide variety of lipids may comprise the non-cationic lipid component of the emulsion surfactant, particularly dipalmitoylphosphatidylcholine, dipalmitoylphosphatidyl-ethanolamine or dioleoylphosphatidylethanolamine in addition to those previously described. In lieu of cationic lipids as described above, lipids bearing cationic polymers such as polylysine or polyarginine may also be included in the lipid surfactant and afford binding of a negatively charged therapeutic, such as genetic material or analogues there of, to the outside of the emulsion particles.

The acoustic contrast substances of the present invention, which are preferably comprised of at least one perfluorocarbon, exhibit a temperature dependent reflectivity when bound to a targert. Perfluorocarbons have been reported to show a linear decrease in ultrasonic velocity with rise in temperature and a decrease in density over temperature ranges of as low as 10°C to as high as 50°C. (Narayana et al., *supra*, 1986). Thus, the temperature dependence of the nanoparticle emulsion or that of its constituent components, such as for example the preferred perfluorocarbon component, can be measured by determining ultrasound velocity of the emulsion or constituent component as is illustrated more fully below in the examples. It is believed that this measurement can also be used to predict the magnitude of change in reflectivity for a given perfluorocarbon component.

The ultrasound target may be an *in vivo* or *in vitro* target and, preferably, a biological material although the target need not be a biological material. The target may be comprised of a surface to which the acoustic contrast substance binds or a three dimensional structure in which the acoustic contrast substance penetrates and binds to portions of the target below the surface.

Preferably, a ligand is incorporated into the acoustic contrast substance to immobilize the acoustic contrast substance to the ultrasound target. The ligand may

be specific for a desired target to allow active targeting. Active targeting refers to ligand-directed, site-specific accumulation of agents to cells, tissues or organs by localization and binding to molecular epitopes, i.e., receptors, lipids, peptides, cell adhesion molecules, polysaccharides, biopolymers, and the like, presented on the surface membranes of cells or within the extracellular or intracellular matrix. A wide variety of ligands can be used including an antibody, a fragment of an antibody, a polypeptide such as small oligopeptide, a large polypeptide or a protein having three dimensional structure, a peptidomimetic, a polysaccharide, an aptamer, a lipid, a nucleic acid, a lectin or a combination thereof. The ligand specifically binds to a cellular epitope or receptor.

The term "ligand" as used herein is intended to refer to a small targeting molecule that binds specifically to another molecule of a biological target separate and distinct from the emulsion particle itself. The reaction does not require nor exclude a molecule that donates or accepts a pair of electrons to form a coordinate covalent bond with a metal atom of a coordination complex. Thus a ligand may be attached covalently for direct-conjugation or noncovalently for indirect conjugation to the surface of the acoustic particle surface.

Avidin-biotin interactions are extremely useful, noncovalent targeting systems that have been incorporated into many biological and analytical systems and selected *in vivo* applications. Avidin has a high affinity for biotin (10^{-15}M) facilitating rapid and stable binding under physiological conditions. Targeted systems utilizing this approach are administered in two or three steps, depending on the formulation. Typically, a biotinylated ligand, such as a monoclonal antibody, is administered first and "pretargeted" to the unique molecular epitopes. Next, avidin is administered, which binds to the biotin moiety of the "pretargeted" ligand. Finally, the biotinylated agent is added and binds to the unoccupied biotin-binding sites remaining on the avidin thereby completing the ligand-avidin-emulsion "sandwich". The avidin-biotin approach can avoid accelerated, premature clearance of targeted agents by the reticuloendothelial system secondary to the presence of surface antibody. Additionally, avidin, with four, independent biotin binding sites provides signal amplification and improves detection sensitivity.

As used herein, the term "biotin agent" or "biotinylated" with respect to conjugation to a biotin agent is intended to include biotin, biocytin and other biotin derivatives and analogs such as biotin amido caproate N-hydroxysuccinimide ester, biotin 4-amidobenzoic acid, biotinamide caproyl hydrazide and other biotin derivatives and conjugates. Other derivatives include biotin-dextran, biotin-disulfide-N-hydroxysuccinimide ester, biotin-6 amido quinoline, biotin hydrazide, *d*-biotin-N hydroxysuccinimide ester, biotin maleimide, *d*-biotin *p*-nitrophenyl ester, biotinylated nucleotides and biotinylated amino acids such as N, ε-biotinyl-l-lysine. The term "avidin agent" or "avidinized" with respect to conjugation to an avidin agent is intended to include avidin, streptavidin and other avidin analogs such as streptavidin or avidin conjugates, highly purified and fractionated species of avidin or streptavidin, and non-amino acid or partial-amino acid variants, recombinant or chemically synthesized avidin.

Targeting ligands may be chemically attached to the surface of acoustic particles by a variety of methods depending upon the nature of the particle surface. Conjugations may be performed before or after the emulsion particle is created depending upon the ligand employed. Direct chemical conjugation of ligands to proteinaceous agents often take advantage of numerous amino-groups (e.g. lysine) inherently present within the surface. Alternatively, functionally active chemical groups such as pyridyldithiopropionate, maleimide or aldehyde may be incorporated into the surface as chemical "hooks" for ligand conjugation after the particles are formed. Another common post-processing approach is to activate surface carboxylates with carbodiimide prior to ligand addition. The selected covalent linking strategy is primarily determined by the chemical nature of the ligand. Monoclonal antibodies and other large proteins may denature under harsh processing conditions; whereas, the bioactivity of carbohydrates, short peptides, aptamers, drugs or peptidomimetics often can be preserved. To ensure high ligand binding integrity and maximize targeted particle avidity flexible polymer spacer arms, e.g. polyethylene glycol or simple caproate bridges, can be inserted between an activated surface functional group and the targeting ligand. These extensions can be 10 nm or longer and minimize interference of ligand binding by particle surface interactions.

Monoclonal antibodies may also be used as site-targeting ligands directed to any of a wide spectrum of molecular epitopes including pathologic molecular epitopes. Immunoglobin-γ (IgG) class monoclonal antibodies have been conjugated to liposomes, emulsions and other microbubble particles to provide active, site-specific targeting. These proteins are symmetric glycoproteins (MW *ca.* 150,000 Daltons) composed of identical pairs of heavy and light chains. Hypervariable regions at the end of each of two arms provide identical antigen-binding domains. A variably sized branched carbohydrate domain is attached to complement-activating regions, and the hinge area contains particularly accessible interchain disulfide bonds that may be reduced to produce smaller fragments.

Bivalent $F(ab')_2$ and monovalent F(ab) fragments can be used as ligands and these are derived from selective cleavage of the whole antibody by pepsin or papain digestion, respectively. Elimination of the Fc region greatly diminishes the immunogenicity of the molecule, diminishes nonspecific liver uptake secondary to bound carbohydrate, and reduces complement activation and resultant antibody-dependent cellular toxicity. Complement fixation and associated cellular cytotoxicity can be detrimental when the targeted site must be preserved or beneficial when recruitment of host killer cells and target-cell destruction is desired (e.g. anti-tumor agents).

Most monoclonal antibodies are of murine origin and are inherently immunogenic to varying extents in other species. Humanization of murine antibodies through genetic engineering has lead to development of chimeric ligands with improved biocompatibility and longer circulatory half-lives. The binding affinity of recombinant antibodies to targeted molecular epitopes can be occasionally improved with selective site-directed mutagenesis of the binding idiotype.

Phage display techniques may be used to produce recombinant human monoclonal antibody fragments against a large range of different antigens without involving antibody-producing animals. In general, cloning creates large genetic libraries of corresponding DNA (cDNA) chains deducted and synthesized by means of the enzyme "reverse transcriptase" from total messenger RNA (mRNA) of human B lymphocytes. Immunoglobulin cDNA chains are amplified by PCR (polymerase chain

reaction) and light and heavy chains specific for a given antigen are introduced into a phagemid vector. Transfection of this phagemid vector into the appropriate bacteria results in the expression of an scFv immunoglobulin molecule on the surface of the bacteriophage. Bacteriophages expressing specific immunoglobulin are selected by repeated immunoadsorption/phage multiplication cycles against desired antigens (e.g., proteins, peptides, nuclear acids, and sugars). Bacteriophages strictly specific to the target antigen are introduced into an appropriate vector, (e.g., Escherichia coli, yeast, cells) and amplified by fermentation to produce large amounts of human antibody fragments with structures very similar to natural antibodies. Phage display techniques have permitted the production of unique ligands for targeting and therapeutic applications.

Polypeptides, like antibodies, may have high specificity and epitope affinity for use as vector molecules for targeted contrast agents. These may be small oligopeptides, having, for example, 5 to 10 amino acid, specific for a unique receptor sequences (such as, for example, the RGD epitope of the platelet GIIbIIIa receptor) or larger, biologically active hormones such as cholecystokinin. Smaller peptides potentially have less inherent immunogenicity than nonhumanized murine antibodies. Peptides or peptide (nonpeptide) analogues of cell adhesion molecules, cytokines, selectins, cadhedrins, Ig superfamily, integrins and the like may be utilized for targeted therapeutic delivery.

Asialoglycoproteins have been used for liver-specific applications due to their high affinity for asialoglycoproteins receptors located uniquely on hepatocytes. Asialoglycoproteins directed agents (primarily magnetic resonance agents conjugated to iron oxides) have been used to detect primary and secondary hepatic tumors as well as benign, diffuse liver disease such as hepatitis. The asialoglycoproteins receptor is highly abundant on hepatocytes, approximately 500,000 per cell, rapidly internalizes and is subsequently recycled to the cell surface. Polysaccharides such as arabinogalactan may also be utilized to localize agents to hepatic targets. Arabinogalactan has multiple terminal arabinose groups that display high affinity for asialoglycoproteins hepatic receptors.

Aptamers are high affinity, high specificity RNA or DNA-based ligands produced by *in vitro* selection experiments (SELEX: systematic evolution of ligands by exponential enrichment). Aptamers are generated from random sequences of 20 to 30 nucleotides, selectively screened by absorption to molecular antigens or cells, and enriched to purify specific high affinity binding ligands. To enhance *in vivo* stability and utility, aptamers are generally chemically modified to impair nuclease digestion and to facilitate conjugation with drugs, labels or particles. Other, simpler chemical bridges often substitute nucleic acids not specifically involved in the ligand interaction. In solution aptamers are unstructured but can fold and enwrap target epitopes providing specific recognition. The unique folding of the nucleic acids around the epitope affords discriminatory intermolecular contacts through hydrogen bonding, electrostatic interaction, stacking, and shape complementarity. In comparison with protein-based ligands, aptamers are stable, are more conducive to heat sterilization, and have lower immunogenicity. Aptamers are currently used to target a number of clinically relevant pathologies including angiogenesis, activated platelets, and solid tumors and their use is increasing. The clinical effectiveness of aptamers as targeting ligands for therapeutic emulsion particles may be dependent upon the impact of the negative surface charge imparted by nucleic acid phosphate groups on clearance rates. Previous research with lipid-based particles suggest that negative zeta potentials markedly decrease liposome circulatory half-life, whereas, neutral or cationic particles have similar, longer systemic persistence.

It is also possible to use what has been referred to as a "primer material" to couple specific binding species to the fluorochemical droplets as disclosed by Millbrath et al. (U.S. Patent No. 5,401,634) for certain *in vitro* applications. As used herein, "primer material" refers to any constituent or derivatized constituent incorporated into the emulsion lipid surfactant layer that could be chemically utilized to form a covalent bond between the particle and a targeting ligand or a component of the targeting ligand such as a subunit thereof.

Thus, the specific binding species (i.e. targeting ligand) may be immobilized on the encapsulating lipid monolayer by direct adsorption to the oil/aqueous interface or using a primer material. A primer material may be any surfactant compatible

compound incorporated in the particle to chemically couple with or adsorb a specific binding or targeting species. The preferred result is achieved by forming an emulsion with an aqueous continuous phase and a biologically active ligand adsorbed or conjugated to the primer material at the interface of the continuous and discontinuous phases. Naturally occurring or synthetic polymers with amine, carboxyl, mercapto, or other functional groups capable of specific reaction with coupling agents and highly charged polymers may be utilized in the coupling process. The specific binding species (e.g. antibody) may be immobilized on the fluorochemical emulsion particle surface by direct adsorption or by chemical coupling. Examples of specific binding species which can be immobilized by direct adsorption include small peptides, peptidomimetics, or polysaccharide-based agents. To make such an emulsion the specific binding species may be suspended or dissolved in the aqueous phase prior to formation of the emulsion. Alternatively, the specific binding species may be added after formation of the emulsion and incubated with gentle agitation at room temperature (25° C) in a pH 7.0 buffer (typically phosphate buffered saline) for 1.2 to 18 hours.

Where the specific binding species is to be coupled to a primer material, conventional coupling techniques may be used. The specific binding species may be covalently bonded to primer material with coupling agents using methods which are known in the art. Primer materials may include phosphatidylethanolamine (PE), N-caproylamine-PE, n-dodecanylamine, phosphatidylthioethanol,N-1,2-diacyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl)butyramide], 1,2-diacyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidomethyl)cyclohexane-carboxylate], 1,2-diacyl-sn-glycero-3-phosphoethanolamine-N-[3-(2-pyridyldithio)propionate], 1,2-diacyl-sn-glycero-3-phosphoethanolamine-N-[PDP(polyethylene glycol)2000], N-succinyl-PE, N-glutaryl-PE, N-dodecanyl-PE, N-biotinyl-PE, or N-caproyl-PE. Additional coupling agents use a carbodiimide such as1-ethyl-3-(3-N,N dimethylaminopropyl)carbodiimide hydrochloride or 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide methyl-p-toluenesulfonate. Other suitable coupling agents include aldehyde coupling agents having either ethylenic unsaturation such as acrolein, methacrolein, or 2-butenal, or having a plurality of aldehyde groups such as glutaraldehyde, propanedial or butanedial. Other coupling

agents include 2-iminothiolane hydrochloride, bifunctional N-hydroxysuccinimide esters such as disuccinimidyl subsrate, disuccinimidyl tartrate, bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone, disuccinimidyl propionate, ethylene glycolbis(succinimidyl succinate); heterobifunctional reagents such as N-(5-azido-2-nitrobenzoyloxy)succinimide, p-azidophenylbromide, p-azidophenylglyoxal,4-fluoro-3-nitrophenylazide, N-hydroxysuccinimidyl-4-azidobenzoate, m-maleimidobenzoyl N-hydroxysuccinimide ester, methyl-4-azidophenylglyoxal, 4-fluoro-3-nitrophenyl azide, N-hydroxysuccinimidyl-4-azidobenzoate hydrochloride, p-nitrophenyl 2-diazo-3,3,3-trifluoropropionate, N-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate, succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, succinimidyl 4-(p-maleimidophenyl)butyrate, N-succinimidyl(4-azidophenyldithio)propionate, N-succinimidyl 3-(2-pyridyldithio)propionate, N-(4-azidophenylthio)phthalamide; homobifunctional reagents such as 1,5-difluoro-2,4-dinitrobenzene, 4,4'-difluoro-3,3'-dinitrodiphenylsulfone, 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene, p-phenylenediisothiocyanate, carbonylbis(L-methionine p-nitrophenyl ester), 4,4'-dithiobisphenylazide, erythritolbiscarbonate and bifunctional imidoesters such as dimethyl adipimidate hydrochloride, dimethyl suberimidate, dimethyl 3,3'-dithiobispropionimidate hydrochloride and the like. Covalent bonding of a specific binding species to the primer material can be carried out with the above reagents by conventional, well-known reactions, for example, in the aqueous solutions at a neutral pH, at temperatures of less than 25 C for 1 hour to overnight.

The emulsions of the present invention may be prepared by various techniques. One method is sonication of a mixture of a fluorochemical liquid and an aqueous solution containing a suitable primer material and/or specific binding species. Generally, these mixtures include a surfactant. Cooling the mixture being emulsified, minimizing the concentration of surfactant, and buffering with a saline buffer will typically maximize both retention of specific binding properties and the coupling capacity of the primer material. These techniques provide excellent emulsions with high activity per unit of absorbed primer material or specific binding species.

When high concentrations of a primer material or specific binding species coated on lipid emulsions, the mixture should be heated during sonication and have a

relatively low ionic strength and moderate to low pH. Too low an ionic strength, too low a pH or too much heat may cause some degradation or loss of all of the useful binding properties of the specific binding species or the coupling capacity of the "primer" material. Careful control and variation of the emulsification conditions can optimize the properties of the primer material or the specific binding species while obtaining high concentrations of coating.

Carbohydrate-bearing lipids may be employed for in vivo targeting, as described in U.S. Pat. No. 4,310,505, the disclosures of which are hereby incorporated herein by reference, in their entirety.

An alternative method of making the emulsions involves directing high pressure streams of mixtures containing the aqueous solution, a primer material or the specific binding species, the fluorocarbon liquid and a surfactant (if any) so that they impact one another to produce emulsions of narrow particle size and distribution. The MICROFLUIDIZER apparatus (Microfluidics, Newton, Mass.) can be used to make the preferred emulsions. The apparatus is also useful to post-process emulsions made by sonication or other conventional methods. Feeding a stream of emulsion droplets through the MICROFLUIDIZER apparatus yields formulations small size and narrow particle size distribution.

Emulsifying and/or solubilizing agents may also be used in conjunction with emulsions. Such agents include, but are not limited to, acacia, cholesterol, diethanolamine, glyceryl monostearate, lanolin alcohols, lecithin, mono- and di-glycerides, mono-ethanolamine, oleic acid, oleyl alcohol, poloxamer, peanut oil, palmitic acid, polyoxyethylene 50 stearate, polyoxyl 35 castor oil, polyoxyl 10 oleyl ether, polyoxyl 20 cetostearyl ether, polyoxyl 40 stearate, polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 80, propylene glycol diacetate, propylene glycol monostearate, sodium lauryl sulfate, sodium stearate, sorbitan mono-laurate, sorbitan mono-oleate, sorbitan mono-palmitate, sorbitan monostearate, stearic acid, trolamine, and emulsifying wax. All lipids with perfluoro fatty acids as a component of the lipid in lieu of the saturated or unsaturated hydrocarbon fatty acids found in lipids of plant or animal origin may be used. Suspending and/or viscosity-increasing agents that may be used with emulsions include, but are not limited to, acacia, agar, alginic acid,

aluminum mono-stearate, bentonite, magma, carbomer 934P, carboxymethylcellulose, calcium and sodium and sodium 12, carrageenan, cellulose, dextrin, gelatin, guar gum, hydroxyethyl cellulose, hydroxypropyl methylcellulose, magnesium aluminum silicate, methylcellulose, pectin, polyethylene oxide, polyvinyl alcohol, povidone, propylene glycol alginate, silicon dioxide, sodium alginate, tragacanth, and xanthum gum.

Useful emulsions may have a wide range of nominal particle diameters, e.g., from as small as about 0.01 μ to as large as 10 μ, preferably about 0.1 to about 0.5 μ in diameter. The emulsion particle sizes can be controlled and varied by modifications of the emulsification techniques and the chemical components. Small size particles are preferred because they circulate longer and tend to be more stable than larger particles.

Targeted therapeutic emulsions may incorporate bioactive agents (e.g drugs, prodrugs, genetic materials, radioactive isotopes, or combinations thereof) in their native form or derivatized with hydrophobic or charged moieties to enhance incorporation or adsorption to the ligand targeted particle. The bioactive agent may be a prodrug, including the prodrugs described, for example, by Sinkyla et al., J. Pharm. Sci., 64:181-210 (1975), in U.S. application Ser. No. 08/851,780 filed May 6, 1997, and in U.S. application Ser. No. 08/887,215 filed Jul. 2, 1997, the disclosures of which are hereby incorporated by reference herein in their entirety.

Such therapeutics may also include, but are not limited to antineoplastic agents, including platinum compounds (e.g., spiroplatin, cisplatin, and carboplatin), methotrexate, fluorouracil, adriamycin, mitomycin, ansamitocin, bleomycin, cytosine arabinoside, arabinosyl adenine, mercaptopolylysine, vincristine, busulfan, chlorambucil, melphalan (e.g., PAM, L-PAM or phenylalanine mustard), mercaptopurine, mitotane, procarbazine hydrochloride dactinomycin (actinomycin D), daunorubicin hydrochloride, doxorubicin hydrochloride, taxol, plicamycin (mithramycin), aminoglutethimide, estramustine phosphate sodium, flutamide, leuprolide acetate, megestrol acetate, tamoxifen citrate, testolactone, trilostane, amsacrine (m-AMSA), asparaginase (L-asparaginase) Erwina asparaginase, interferon α-2a, interferon α-2b, teniposide (VM-26), vinblastine sulfate (VLB), vincristine sulfate, bleomycin, bleomycin sulfate, methotrexate, adriamycin, arabinosyl,

hydroxyurea,procarbazine, dacarbazine, mitotic inhibitors such as etoposide and other vinca alkaloids; radiopharmaceuticals such as but not limited to radioactive iodine, samarium, strontium cobalt, yittrium and the like; protein and nonprotein natural products or analogues/mimetics thereof including hormones such as but not limited to growth hormone, somatostatin, prolactin, thyroid, steroids, androgens, progestins, estrogens and antiestrogens; analgesics including but not limited to antirheumatics, such as auranofin, methotrexate, azathioprine, sulfazalazine, leflunomide, hydrochloroquine, and etanercept; muscle relaxants such as baclofen, dantrolene, carisoprodol, diazepam, metaxalone, cyclobenzaprine, chlorzoxazone, tizanidine; narcotic agonists such as codeine, fentanyl, hydromorphone, lleavorphanol, meperidine, methadone, morphine, oxycodone, oxymorphone, propoxyphene; narcotic agonist-antagonists such as buprenorphine, butorphanol, dezocine, nalbuphine, pentazocine; narcotic antagonists such as nalmefene and naloxone, other analgesics including ASA, acetominophen, tramadol, or combinations thereof; nonsteroidal anti-inflammatories including but not limited to celecoxib, diclofenac, diflunisal, etodolac, fenoprofen, flurbiprofen, ibuprofen, indomethacin, ketoprofen, ketorolac, naproxen, oxaproxen, rofecoxib, salisalate, suldindac, tolmetin; anesthetic and sedatives such as etomidate, fentanyl, ketamine, methohexital, propofol, sufentanil, thiopental, and the like; neuromuscular blockers such as but not limited to pancuronium, atracurium, cisatracurium, rocuronium, succinylcholine, vercuronium; antimicrobials including aminoglycosides, antifungal agents including amphotericin B, clotrimazole, fluconazole, flucytosine, griseofulvin, itraconazole, ketoconazole, nystatin, and terbinafine; anti-helmintics; antimalarials, such as chloroquine, doxycycline, mefloquine, primaquine, quinine; antimycobacterial including dapsone, ethambutol, ethionamide, isoniazid, pyrazinamide, rifabutin, rifampin, rifapentine; antiparasitic agents including albendazole, atovaquone, iodoquinol, ivermectin,mebendazole, metronidazole, pentamidine, praziquantel, pyrantel, pyrimethamine, thiabendazole; antiviral agents including abacavir, didanosine, lamivudine, stavudine, zalcitabine, zidovudine as well as protease inhibitors such as indinavir and related compounds, anti-CMV agents including but not limited to cidofovir, foscarnet, and ganciclovir; antiherpetic agents including amatadine, rimantadine, zanamivir; interferons, ribavirin, rebetron; carbapenems, cephalosporins, fluoroquinones, macrolides,

penicillins, sulfonamides, tetracyclines, and other antimicrobials including aztreonam, chloramphenicol, fosfomycin, furazolidone, nalidixic acid, nitrofurantoin, vancomycin. and the like; nitrates, antihypertensives including diuretics, beta blockers, calcium channel blockers, angiotensin converting enzyme inhibitors, angiotensin receptor antagonists, antiadrenergic agents, anti-dysrhythmics, antihyperlipidemic agents, antiplatelet compounds, pressors, thrombolytics, acne preparations, antipsoriatics; corticosteroids; androgens, anabolic steroids, bisphosphonates; sulfonoureas and other antidiabetic agents; gout related medicants; antihistamines, antitussive, decongestants, and expectorants; antiulcer medicants including antacids, 5-HT receptor antagonists, H2-antagonists, bismuth compounds, proton pump inhibitors, laxatives, octreotide and its analogues/mimetics; anticoagulants; immunization antigens, immunoglobins, immunosuppressive agents; anticonvulsants, 5-HT receptor agonists, other migraine therapies; parkinsonian agents including anticholinergics, and dopaminergics; estrogens, GnRH agonists, progestins, estrogen receptor modulators, tocolytics, uterotnics,thyroid agents such as iodine products and anti-thyroid agents; blood products such as parenteral iron, hemin, hematoporphyrins and their derivatives.

Genetic material, includes, for example, nucleic acids, RNA and DNA, of either natural or synthetic origin, including recombinant RNA and DNA and antisense RNA and DNA; hammerhead RNA, ribozymes, hammerhead ribozymes, antigene nucleic acids, both single and double stranded RNA and DNA and analogs thereof, ribooligonucleotides, antisense ribooligonucleotides, deoxyribooligonucleotides, and antisense deoxyribooligonucleotides. Other types of genetic material that may be used include, for example, genes carried on expression vectors such as plasmids, phagemids, cosmids, yeast artificial chromosomes, and defective or "helper" viruses, antigene nucleic acids, both single and double stranded RNA and DNA and analogs thereof, such as phosphorothioate and phosphorodithioate oligodeoxynucleotides. Additionally, the genetic material may be combined, for example, with proteins or other polymers.

As we have previously described, the emulsion nanoparticles may incorporate on the particle paramagnetic or super paramagnetic elements including but not limited

to gadolinium, magnesium, iron, manganese in their native or in a chemically complexed form. Similarly, radioactive nuclides including positron-emitters, gamma-emitters, beta-emitters, alpha-emitters in their native or chemically-complexed form may be included on or in the particles. Adding of these moieties permits the additional use of other clinical imaging modalities such as magnetic resonance imaging, positron emission tomography, and nuclear medicine imaging techniques in conjunction with temperature enhanced ultrasonic imaging. Moreover, the inclusion of metal ions in or on the formulation may be utilized as "seeds" to augment or implement local hyperthermia.

In addition, optical imaging, which refers to the production of visible representations of tissue or regions of a patient produced by irradiating those tissues or regions of a patient with electromagnetic energy in the spectral range between ultraviolet and infrared, and analyzing either the reflected, scattered, absorbed and/or fluorescent energy produced as a result of the irradiation, may be combined with the enhanced acoustic reflectivity of temperature-dependent targeted emulsions. Examples of optical imaging include, but are not limited to, visible photography and variations thereof, ultraviolet images, infrared images, fluorimetry, holography, visible microscopy, fluorescent microscopy, spectrophotometry, spectroscopy, fluorescence polarization and the like.

Photoactive agents, i.e. compounds or materials that are active in light or that responds to light, including, for example, chromophores (e.g., materials that absorb light at a given wavelength), fluorophores (e.g., materials that emit light at a given wavelength), photosensitizers (e.g., materials that can cause necrosis of tissue and/or cell death in vitro and/or in vivo), fluorescent materials, phosphorescent materials and the like, that may be used in diagnostic or therapeutic applications. "Light" refers to all sources of light including the ultraviolet (UV) region, the visible region and/or the infrared (IR) region of the spectrum. Suitable photoactive agents that may be used in the present invention have been described by others (Unger et al 6,123,923) are incorporated by reference herein and include but are not limited to, for example, fluoresceins, indocyanine green, rhodamine, triphenylmethines, polymethines, cyanines, fullerenes, oxatellurazoles, verdins, rhodins, perphycenes, sapphyrins,

rubyrins, cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoate, cholesteryl 12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-dodecanate, cholesteryl cis-parinarate, cholesteryl 3-((6-phenyl)-1,3,5-hexatrienyl)phenyl-proprionate, cholesteryl 1-pyrenebutyrate, cholesteryl 1-pyrenedecanoate, cholesteryl 1-pyrenehexanoate, 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3.be ta.-ol, 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3.be ta.-yl cis-9-octadecenoate, 1-pyrenemethyl3-(hydroxy-22,23-bisnor-5-cholenate, 1-pyrene-methyl 3.beta.-(cis-9-octadecenoyloxy)-22,23-bisnor-5-cholenate, acridine orange 10-dodecyl bromide, acridine orange 10-nonyl bromide, 4-(N,N-dimethyl-N-tetradecylammonium)-methyl-7-hydroxycoumarin) chloride, 5-dodecanoylaminofluorescein, 5-dodecanoyl-aminofluorescein-bis-4,5-dimethoxy-2-nitrobenzyl ether, 2-dodecylresorufin, fluorescein octadecyl ester, 4-heptadecyl-7-hydroxycoumarin, 5-hexadecanoylaminoeosin, 5-hexadecanoylaminofluorescein,5-octadecanoylaminofluorescein, N-octadecyl-N'-(5-(fluoresceinyl))thiourea, octadecyl rhodamine B chloride, 2-(3-(diphenylhexatrienyl)-propanoyl)-1-hexadecanoyl-sn-glycero- 3-phosphocholine, 6-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic acid, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate, 12-(9-anthroyloxy)oleic acid, 5-butyl-4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-3-nonanoic acid, N-(lissamine.TM. rhodamine B sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt, phenylglyoxal monohydrate, naphthalene-2,3-dicarboxaldehyde, 8-bromomethyl-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indace ne, o-phthaldialdehyde, lissamine.TM. rhodamine B sulfonyl chloride, 2 ',7'-difluorofluorescein, 9-anthronitrile, 1-pyrenesulfonyl chloride, 4-(4-(dihexadecylamino)-styryl)-N-methylpyridinium iodide, chlorins, such as chlorin, chlorin e6, bonellin, mono-L-aspartyl chlorin e6, mesochlorin, mesotetraphenylisobacteriochlorin, and mesotetraphenylbacteriochlorin, hypocrellin B, purpurins, such as octaethylpurpurin, zinc(II) etiopurpurin, tin(IV) etiopurpurin and tin ethyl etiopurpurin, lutetium texaphyrin, photofrin, metalloporphyrins, protoporphyrin IX, tin protoporphyrin, benzoporphyrin, haematoporphyrin, phthalocyanines, naphthocyanines, merocyanines, lanthanide complexes, silicon

phthalocyanine, zinc phthalocyanine, aluminum phthalocyanine, Ge octabutyoxyphthalocyanines, methyl pheophorbide-.alpha.-(hexyl-ether), porphycenes, ketochlorins, sulfonated tetraphenylporphines, .delta.-aminolevulinic acid, texaphyrins, including, for example, 1,2-dinitro-4-hydroxy-5-methoxybenzene, 1,2-dinitro-4-(1-hydroxyhexyl)oxy-5-methoxybenzene, 4-(1-hydroxyhexyl)oxy-5methoxy-1,2-phenylenediamine, and texaphyrin-metal chelates, including the metals Y(III), Mn(II), Mn(III), Fe(II), Fe(III) and the lanthanide metals Gd(III), Dy(III), Eu(III), La(III), Lu(III) and Tb(III), chlorophyll, carotenoids, flavonoids, bilins, phytochromes, phycobilins, phycoerythrins, phycocyanines, retinoic acids, retinoins, retinates, or combinations of any of the above.

One skilled in the art will readily recognize or can readily determine which of the above compounds are, for example, fluorescent materials and/or photosensitizers. LISSAMINE. is the trademark for N-ethyl-N-[4-[[4-[ethyl [(3-sulfophenyl)methyl]-amino]phenyl](4-sulfophenyl)-methylene]-2,5-cyclohexadien-1-ylidene]-3-sulfobenzene-methanaminium hydroxide, inner salt, disodium salt and/or ethyl[4-[p[ethyl(m-sulfobenzyl)amino]-.alpha.-(p-sulfophenyl)benzylidene]- 2,5-cyclohexadien-1-ylidene](m-sulfobenzyl)ammonium hydroxide inner salt disodium salt (commercially available from Molecular Probes, Inc., Eugene, Oreg.). Other suitable photoactive agents for use in the present invention include those described in U.S. Pat. No. 4,935,498, the disclosure of which is hereby incorporated by reference herein in its entirety, such as a dysprosium complex of 4,5,9,24-tetraethyl-16-(1-hydroxyhexyl)oxy-17 methoxypentaazapentacyclo-(2 0.2.1.1.sup.3,6.1. sup.8,11.0.sup.14,19)-heptacosa-1,3,5,7,9,11(27),12,14,16,18,20,22(25),23-tridecaene and dysprosium complex of 2-cyanoethyl-N,N-diisopropyl-6-(4,5,9,24-tetraethyl-17-methoxypentaazapent acyclo-(20.2.1.1 .sup.3,6.1.sup.8,11.0.sup.14,19)-heptacosa-1,3,5,7,9,11(27), 12,14,16,18,20,22(25),23-tridecaene-16-(1-oxy)hexylphosphoramidite.

In addition, certain ligands, such as, for example, antibodies, peptide fragments, or mimetics of a biologically active ligand may contribute to the inherent therapeutic effects, either as an antagonistic or agonistic, when bound to specific epitopes. As an example, antibody against $\alpha_v\beta_3$ integrin on neovascular endothelial

cells has been shown to transiently inhibit growth and metastasis of solid tumors. The efficacy of therapeutic emulsion particles directed to the $\alpha_v\beta_3$ integrin may result from the improved antagonistic action of the targeting ligand in addition to the effect of the therapeutic agents incorporated and delivered by particle itself.

Changes in temperature of the acoustic imaging substance bound to the target can involve increases or decreases in temperature. In embodiments in which the temperature of the acoustic imaging substance is increased, an energy source is used to increase the energy and an increase in acoustic reflectivity is measured.

Local hyperthermia may be induced at the site of targeted nanoparticle emulsions by a variety of modalities including but not limited to ultrasound, shortwave, microwave, magnetic radiation, electromagnetic energies or combination thereof. Such energy may be applied noninvasively by external systems or more invasively by catheter systems. Yang et al. (*Med Biol Eng Comput* 17:518-24, 1979) have shown that microwaves (e.g. 2500 MHz) provide excellent superficial heating to the skin, 900 MHz radiation induces pronounced temperature rises in the musculature and shortwaves at 27 MHz produce a wide plateau of elevated temperature in the muscle layers. These investigators have shown how wave frequency, power, ambient conditions, vasodilation and core boundary conditions can be varied to control local hyperthermia. Others have report the use of ferrimagnetic resonance of a ferrite-impregnated medium as the heating target which is placed internally and heated externally by radiated microwaves. This increased the depth of heating by up to 50% versus nonresonance techniques.

Minimally invasive thermal therapy is a currently used cancer treatment for treating solid tumors and the procedure can also provide the local hyperthemia for temperature-dependent acoustic contrast enhancement of ligand-targeted emulsions. Such approaches impart high temperatures over short time-frames (from microseconds to minutes). Interstitial heating localize the target tissue volume and minimize the effect of heating on surrounding normal tissues. Interstitial heating energy is typically delivered by laser light, microwaves, or ultrasound. The choice of energy source depends on the target site, applicator geometry, and blood perfusion to the site. The decrease of energy with distance due to applicator geometry is more important for

targets close to the applicator while the fall-off of energy due to attenuation is more important further from the energy source. Thus, laser light, which is highly scattered in tissues, is appropriately applied to targets close to the applicator and ultrasound or microwaves are better suited for heating deeper structures (Skinner et al., *Phys Med Biol 43*:3535-47, 1998).

The heating effect is believed to is believed to require intensities that are preferably greater than 0.1 W/cm2 (typical ultrasound imaging fields). Also, the prefered intensity level is less than those used for high intensity focused ultrasound (such as, for example, approximately 2000 W/cm2). Additional preferences are that nondestructive pulses and intensity levels be used to leave the tissue itself unharmed. It is believed that high intensity but short pulse durations will also be useful for this application in the range of microseconds to milliseconds, depending on transducer characteristics, depth of tissue interrogated, tissue attenuation,beam dispersion and other physical features. These parameters are themselves distinguishable from more prevalent high intensity focused ultrasound techniques for therapeutic ultrasound uses.

In other instances, the methods of the present invention involve decreasing the temperature of the acoustic imaging substance and measurement of the decrease in reflectivity. The decrease in temperature can be produced by an energy absorbing component such as a cryogenic device for use in cryotherapy. Cryotherapy, which is also sometimes referred to as cryosurgery, is well known in the art involving the use of a liquid nitrogen or liquid argon in a probe as an energy absorber such that the extreme cold kills cancer cells contacted by the probe. (see for example Lee et al., *Urology 54*:135-40, 1999). The methods of the present invention provide an approach for targeting of the energy absorber based upon the decrease in acoustic reflectivity of the acoustic imaging substance bound to the target tissue as well as providing an approach for monitoring the decrease in temperature.

In certain embodiments, the methods of the present invention involve the measurement of the acoustic reflectivity of the acoustic imaging substance upon changing the temperature of the acoustic imaging substance compared to the acoustic reflectivity in absence of the energy source or the energy absorber which changes the temperature of the acoustic imaging substance. The measurement in absence of the

energy source or energy absorber can be performed either before or after the energy change. Thus it is possible to measure acoustic reflectivity under control conditions prior to and then immediately after temperature change. Alternatively, the acoustic reflectivity can be measured upon achieving the energy change and then some time later after the energy change has dissipated to return the target to a temperature approaching that prior to the energy change.

The measurement of the difference in acoustic reflectivity can be represented in a number of ways, for example, as a digital numeric representation of the reflectivity difference, as a differential two or three dimensional image, as a colorized differential image and the like.

The present invention also includes devices for performing the methods. The devices measure changes in temperature of a target to which a temperature-sensitive acoustic imaging substance is bound. The device comprises a component configured to change the temperature of the acoustic imaging substance, an ultrasound source configured to transmit acoustic energy to the target, an ultrasound detecting component configured to measure acoustic reflectivity of the surface and a comparator which determines acoustic reflectivity of the target upon changing temperature relative to acoustic reflectivity of the target in absence of changing temperature. The ultrasound transmitting component and ultrasound detecting component are preferably comprised of at least one ultrasound piezoelectric transducer. The temperature changing component can be an energy source, such as a source for ultrasound, shortwave, microwave, magnetic radiation, electromagnetic energy or an energy absorber such as a cryogenic component comprising circulated liquid nitrogen or liquid argon. Preferably, the energy changing component is in the form of a probe.

The comparator component of the device provides the differential measurement in the absence and presence of the temperature change produced by the energy changing component. In one embodiment, the comparator comprises an image processor for producing a differential image based upon a subtraction of the acoustic reflectivity images produced in absence and presence of the energy change. The image processor may include an image frame storage component and/or electronic

components such as computer hardware and software components to produce the subtraction image.

Preferred embodiments of the invention are described in the following examples. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the examples.

EXAMPLE 1

This example illustrates the measurement of temperature dependence of ultrasound velocity in perfluorooctane, perfluorodichlorooctane and perfluorooctylbromide.

Ultrasound velocities were determined using a 25 MHz, Panametrics V324 spherically focused transducer. Measurements were made for perfluorooctane, perfluorodichlorooctane or perfluorooctylbromide at discrete temperatures by placing 8 mL of fluorocarbon in a sealed, vertically mounted sample chamber in heated water bath. The back of the chamber consisted of a stainless steel reflector, which extended past the fully enclosed well to allow for water-path and sample-path measurements. The chamber was mounted so that the stainless steel reflector was perpendicular to the insonifying beam.

The times of flight from the transducer to front wall of the chamber and from the transducer to the stainless steel plate were determined for nine independent locations over the sample. The speeds of sound were then averaged together for each temperature. The temperature was changed by two-degree increments from 25 to 47 C by heating the surrounding water bath and allowing time for the sample to reach equilibrium (typically 20 to 25 minutes). Speed of sound was calculated using a previously published algorithm.(Kuo et al., *J. Acoust. Soc. Am. 88*:1679-82, 1990)

Speed of sound measurements are summarized in Figure 1. As has been previously reported for other liquid perfluorocarbons, the speed of sound showed a linear decrease with increasing temperature for each of the perfluorocarbons.

EXAMPLE 2

This example illustrates the preparation of a biotinylated microemulsion for avidin-biotin targeting.

A biotinylated emulsion was prepared by incorporating biotinylated phosphatidylethanolamine into the outer lipid monolayer of a perfluorocarbon microemulsion. The microemulsion was prepared containing perfluorooctane (40% w/v, 3M), vegetable oil (2% w/v) a surfactant co-mixture (2.0%, w/v) and glycerin (1.7%, w/v) in water as follows. The surfactant co-mixture was prepared by dissolving 64 mole% lecithin (Pharmacia, Inc), 35 mole% cholesterol (Sigma Chemical Co.) and 1 mole% N-(6-(biotinoyl)amino) hexanoyl)-dipalmitoyl-L-alpha-phosphatidyl-ethanolamine, Pierce, Inc.) in chloroform. The chloroform-lipid mixture was evaporated under reduced pressure, dried in a 50°C vacuum oven overnight and dispersed into water by sonication. The suspension was then transferred into a blender cup (Dynamics Corporation of America) with the fluorocarbon, vegetable oil, glycerin and distilled, deionized water and emulsified for 30 to 60 seconds. The emulsified mixture was transferred to a MICROFLUIDICS emulsifier (Microfluidics Co.) and continuously processed at 20,000 PSI for three minutes. The completed emulsion was vialed, blanketed with nitrogen and sealed with stopper crimp seal until use. Particle sizes were determined in triplicate at 37°C with a laser light scattering submicron particle size analyzer (Malvern Zetasizer 4, Malvern Instruments Ltd, Southborough, MA)., which indicated a narrow size distribution with average particle diameter less than 400 nm.

EXAMPLE 3

This example illustrates a method which can be used to prepare an emulsion in which the nanoparticles are conjugated with an F(ab) fragment.

Targeting of emulsions can be achieved by direct chemical conjugation of an antibody to the nanoparticle through a primer material incorporated into the lipid monolayer. The perfluorocarbon nanoparticle contrast agent is prepared as described in Example 1.

F(ab) fragments are generated and isolated using an immunopure F(ab) preparation kit (Pierce, Rockford, IL). Briefly, IgG is dialyzed into 20mM phosphate/10mM EDTA buffer (pH 7.0), concentrated to 20 mg/ml and digested by immobilized papain. Solubilized F(ab) is purified from Fc fragments and undigested IgG protein using a protein A column. F(ab) fragments are purified from excess cysteine using a G25-150 column and deoxygenated phosphate buffer (pH 6.7). Fraction identity is confirmed by routine SDS-PAGE procedures.

F(ab) fractions are pooled and combined with the primer-derivatized emulsion (1-2 mg F(ab)/ml of emulsion). The mixture is adjusted to pH 6.7, sealed under nitrogen and allowed to react overnight at ambient temperatures with gentle, continuous mixing. The mixture may be subsequently dialyzed with a 300,000 MWCO Spectra/Por DispoDialyzer (Laguna Hills, CA) against 10mM phosphate buffer (pH 7.2) to remove unconjugated F(ab) fragments. The final emulsion is vialed under nitrogen and stored at 4° C until use. A nonspecific control emulsion may be prepared using the control, irrelevant IgG F(ab) fragments in the above protocol.

EXAMPLE 4

This example illustrates the temperature-dependent targeting of nitrocellulose membranes using microemulsion ultrasound contrast agent bound to the target with avidin-biotin conjugation.

The ultrasonic data acquisition set-up and analysis to measure acoustic reflectance backscatter was as follows. A 25MHz, spherically focused transducer (0.63 mm diameter, 2.54mm focal length, Panametrics V324) was mounted on a gantry consisting of three orthogonal sleds. The transducer was translated in a raster scan format by a computer controlled motion controller (Aerotech Unidex 12) with 100 μm resolution. The pulses sent to the motor from the motion controller were counted in a digital counter (National Instruments PCI-1200) and then a trigger was generated for a digital delay generator (Stanford Research Systems DG535). The delay generator then sent a trigger for the pulser (Panametrics 5900) and for the digitizing oscilloscope (Hewlett-Packard 54510B), as well as a delayed trigger for the real time digitizer (Tektronix RTD720A). Traces representing the backscattered

ultrasonic wave were captured on the fly as the transducer was scanned over the surface of the clot in a 68 x 68 (6.8 mm x 6.8 mm) grid at 100 μm resolution. The traces were then transferred from the real time digitizer to the controlling computer (Apple Power Macintosh 7300) over GPIB for image reconstruction and data storage. Acquisition typically took about 4 minutes per scan.

The sample chamber consisted of a fully enclosed well with an acoustic aperture to allow insonification of the sample. The chamber was attached through two ports to silicone tubing (Masterflex Platinum, I.D.=1/8") that allowed perfusion of the contrast agent over the sample. A roller pump (Masterflex, Cole-Parmer Inc.) was used to drive the flow at a rate of 20mL/min. The flow system was filled with 20 mL of 50mM phosphate buffer. The sample chamber and enclosed sample were positioned vertically so that no passive settling of the contrast agent could occur. After initial location of the sample, a bolus of 100μL of the contrast agent was delivered through an injection port and ultrasonic monitoring was performed initially and after 60 minutes of exposure. The chamber and tubing were then flushed with phosphate buffer.

After confirmation of successful targeting of the contrast agent by imaging, the temperature was varied in 5° C increments from 27 to 47° C using an immersion heater controlled by a temperature regulator (DigiSense, Cole-Parmer Inc.). The entire water bath was placed on top of a magnetic stirrer plate to allow for adequate mixing and homogenous temperature distribution throughout the bath. At each temperature point, the focus of the transducer was determined by observing the reflection from a steel plate. The front wall of the sample was then placed at the focus of the transducer.

The reflected ultrasonic signals were full-wave rectified and used to render a peak-detected c-scan image so that a user-defined region of interest could be drawn around the clot or nitrocellulose sample. The signals representing the reflection of the interrogating wave of ultrasound from the surface of the sample were isolated with a rectangular windowing function. The placement of the window was carefully controlled in the case of the thin nitrocellulose samples by an automatic algorithm that placed the end of the window midway between the front and back wall echo of the

nitrocellulose paper. The isolated signal was then fast Fourier transformed and the average power over the usable bandwidth (17 to 35 MHz, as determined by 10dB down points) was calculated in the logarithmic domain. This "integrated power" was then sorted for all of the points in the region of interest and the brightest 100 points were retained for analysis. The integrated power determined at every point in the scan was also used to render images of the change in ultrasonic enhancement of the clot. The frequency-dependent reflection enhancement was averaged for the 100 brightest points and then normalized by subtracting the reflection enhancement for the control scan. This process was performed for each sample.

Nitrocellulose membranes were prepared as follows for avidin-biotin targeting of temperature-dependent targeted emulsions in vitro. Flat nitrocellulose membranes were prepared for contrast binding with a diaminohexane spacer and activated with glutaraldehyde for protein conjugation. Nitrocellulose discs (2.5 cm diameter) were immersed in 1,6 diaminohexane (2.5% w/v, pH 11.9) for 60 minutes under constant rotary agitation. The membranes were next washed under constant agitation for approximately 12 hours in 1M acetic acid followed by 12 hours in ultrapure water with several changes of each medium. The diaminoalkane-modified nitrocellulose membranes were then exposed to 1% glutaraldehyde in 0.5 M $NaHCO_3$ / Na_2CO_3, pH 10, for 15 minutes followed by a three hour wash in several changes of ultrapure water. The diaminohexane-modified, glutaraldehyde-activated nitrocellulose membranes were stored dry at 4° C until use.

Avidin (50 μg) dissolved in 0.1 M phosphate buffered saline (PBS) (pH 7.2-7.4) was spotted and air-dried dropwise onto the center of each membrane with a microliter syringe and allowed to dry. Next, each membrane was washed with three, five-minute changes of PBS-0.1% Tween 20. Dehydrated milk powder suspended in PBS-0.1% Tween 20 was used to block glutaraldehyde activated protein binding sites left unoccupied after the application of avidin for 20 minutes. Excess protein was removed with three, five minute isotonic, PBS washes.

Five avidin-derivatized and five control nitrocellulose discs were utilized for exposure to biotinylated perfluorooctane particles. After confirmation of successful targeting of the contrast agent by acoustic imaging, the targeted sample temperature

was varied in 5° C increments from 27 to 47° C using an immersion heater controlled by a temperature regulator (DigiSense, Cole-Parmer Inc.). The entire water bath was placed on top of a magnetic stirrer plate to allow for adequate mixing and homogenous temperature distribution throughout the bath. At each temperature point, the focus of the transducer was determined by observing the reflection from a steel plate. The front wall of the sample was then placed at the focus of the transducer.

Figure 2 demonstrates the change of ultrasonic reflected power with temperature for targeted and control nitrocellulose membranes. The membranes were spotted with a single drop of avidin resulting in the almost perfectly circular feature. The pictures shows the change in reflected enhancement for the transition from the baseline temperature of 27 C to each individual temperature points. Darker areas represent areas of larger changes in enhancement and can be seen to increase substantially for targeted nitrocellulose in comparison to the control nitrocellulose.

The 100 brightest sites for each nitrocellulose membrane sample were selected to determine the average frequency-dependent reflection enhancement. Each curve was then normalized to the respective reflection enhancement from the control nitrocellulose membrane at the same temperature.

The progressive increase in ultrasonic backscatter enhancement with temperature was further quantified in Figure 3 with the bandwidth (17 to 34 MHz) limited average. The increased impedance mismatch between the bound nanoparticles and the substrate that occurs with increased temperature results in improved reflection enhancement from targeted substrates. The correlation between contrast enhancement and temperature for nitrocellulose (R=0.95) is excellent. Figures 3 quantify the changes in reflection enhancement as a function of temperature. Overall, the increase in enhancement is 0.08 dB/C for nitrocellulose.

EXAMPLE 5

This example illustrates the temperature-dependent targeting of plasma-rich plasma clots on nitrocellulose membranes using microemulsion ultrasound contrast agent bound to the target with avidin-biotin conjugation.

Biotinylated 1H10 Antibody was prepared using the EZ-LINK Sulfo-NHS-LC-Biotinylation Kit. Briefly, 2 to 10 mg of antibody in 1 ml of phosphate buffered saline is combined with Sulfo-NHS-LC-Biotin in distilled water to afford a 12 to 20-fold molar excess of reagent to antibody. The solution was then incubated at room temperature for 30 minutes. Biotinylated antibody was separated from reagents using a 10 ml desalting column. Equilibration and elution was performed with phosphate buffered saline. Fractions of eluate were collected and UV absorbance measured at 280 nm with a spectrophotometer. Fractions containing antibody were stored at 4°C until use.

Plasma clots were produced on nitrocellulose membrane surfaces by combining human citrated plasma (375 µl) and 100-mM calcium chloride (25µl) with three units of thrombin in a plastic mold placed on the membranes. The plasma was allowed to coagulate slowly at ambient temperature and then transferred to PBS until exposure to the control or targeted contrast system.

Clots (n=9) were incubated with the biotinylated 1H10 antibody overnight in PBS in a 4° C cold room under gentle agitation. The clots were then rinsed and exposed to100µg of avidin under gentle agitation for one hour at room temperature. The clots were then re-rinsed and ready for exposure to contrast agent as described in Example 4. Control clots (n=7), not pretargeted with biotinylated antibody or avidin, were exposed in identical manner to the biotinylated contrast agent.

Measurement of acoustic reflectivity was performed as in Example 4. The inherently poor echogenicity of the native human plasma clots is shown in the left portion of the Figure 4. The figure illustrates two images of the integrated reflected power of a plasma clot before and after delivery of the contrast agent, with the grayscale representing a logarithmic depiction of the reflection enhancement. Darker areas on the right picture represent areas where the enhancement of the clot is increased by the presence of site-targeted ultrasonic contrast agent.

The same plasma clot depicted in Figure 4 is also shown in Figure 5 where the grayscale now represents the change in enhancement brought about by increases in temperature in 5° C increments from the 27° C baseline. As in the case of targeted nitrocellulose, targeted human plasma clots show substantial increase in echogenicity

with temperature while the control clots (unexposed to contrast agent) show little change over this temperature range. Figure 6 shows the results of the reflection enhancement for the 100 brightest sites on the clots averaged over the usable bandwidth of the transducer (17 to 34 MHz). A linear fit to this plot of reflection enhancement vs. temperature yields a correlation coefficient of R=0.99 and a slope of 0.21 dB/C.

In comparison, the control nitrocellulose and control human plasma clots both show only a small change in reflected power as the temperature increases. The targeted or enhanced substrates both exhibit detectable changes in enhancement with as little as a 5° C change in temperature. This differential acoustic reflectivity response can be utilized to segment between contrast targeted and nontargeted surfaces and enhance image presentations through colorization, digital subtraction or similar techniques. The increased sensitivity of targeted tissue detection as well as capability for improved presentation to and recognition by clinical readers could markedly improve the recognition of pathologies that might have otherwise have been unappreciated.

EXAMPLE 6

This example illustrates the lack of hystersis of temperature-dependent change in acoustic reflectivity of avidin-biotin targeted fluorocarbon emulsion bound to human plasma clot.

To test for the reversibility of the temperature effect, two human plasma clot samples were heated and then cooled and the reflection enhancement recorded for each temperature point using techniques described in example 3. The hysteresis curves are plotted in Figure 7. Within the error bars of the measurements, the magnitude of enhancement was identical at each temperature, independent of the direction of temperature change. Given the boiling point of the selected fluorocarbon, i.e. perfluorooctane (105° C), and the heat-dissipating effect of the 37° C water bath in which the samples were insonified, these results confirm that enhancement or diminishment of acoustic reflectivity of targeted tissues results from temperature-

dependent changes in acoustic impedence of the bound emulsion particles and not a phase-transition of the fluorocarbon to a gaseous state.

EXAMPLE 7

This example illustrates methodology and instrumentation that can be used in applying the present invention to ultrasound imaging in conjunction with hyperthermia therapy in specifically detecting, localizing and defining the morphology of the tumor burden and in monitoring the hyperthermia.

Hyperthermia in various forms is now used in conjunction with radiation oncology as an augmentive treatment for various cancers, particularly those less than 8 cm in depth. One device currently used for this application is the Sonotherm® 1000 produced by Labthermics Technologies, Champaign, IL. Optimal tissue hyperthermia depends on heating the tumor with minimum treatment of surrounding normal tissues. SONOTHERM 1000 has the capability to segment a specified tissue volume into small cubic treatment voxels. By adjusting the frequency and intensity of different elements of the ultrasound array, "optimal" heating patterns are achieved.

The keys to all this regimen, especially with regard to difficult to distinguish small tumors, are (1) the precise localization and morphologic delineation of the tumor in a three-dimensional volume space and (2) noninvasive thermometry of the tissue heating process to ensure tumor kill and sparing of normal collateral tissues.

The temperature-dependent ultrasound contrast agents of the present invention will greatly enhance the high-resolution detection, localization and mapping of tumors in two-dimensional or three-dimensional space, particularly when the cancer is small or the background in inherently acoustically reflective. This is achieved through the differential ultrasonic response of nanoparticle-targeted and surrounding normal tissues. In addition, the temperature-dependent changes in acoustic backscatter could be used an internal thermometry, assuring that targeted tissues are heated to appropriate levels while of other tissue heating is minimized. This high resolution, noninvasive thermometry may be constantly displayed in real-time using a scaled color map to allow the operator to monitor tissue temperatures and manually adjust

the hyperthermetry protocol. Alternatively, ultrasonic beam adjustments may be automatically implemented "on-the-fly" by the hyperthermia machine through self-monitoring algorithms.

Pathologies, such as tumors, are often difficult to differentiate from normal tissues with routine ultrasound and benefit from specific contrast enhancement. Unfortunately, the high acoustic reflectivity of adjacent tissues may often diminish the magnitude of contrast enhancement achieved, even with targeted molecular imaging agents. The opportunity to use temperature dependent, targeted molecular contrast agents further provides a unique mechanism for differentiating normal from pathologic tissues by increasing the acoustic signal from targeted versus normal surrounding tissues. This feature will allow refined segmentation and localization of tumors, particularly small tumors, for biopsy or external radiation or hyperthermic therapy. Improved localization affords more precise focusing of external therapeutic energy sources, maximizing the efficiency of treatment and minimizing colateral damage. In addition, the magnitude of increased acoustic backscatter relative to surrounding tissues provides noninvasive thermometry. The proper level of heating at the target site may be monitored and controlled manually or by self-regulating systems within hyperthermia instrumentation. Technical advantages can be immediately envisioned for the treatment of breast cancer, malignant melanoma, sarcomas, lymphomas, head and neck cancers, and soon more deeply positioned tumors such as colon, cervical, uterine, hepatic, pancreatic, gastric and the like.

For instance, a patient or animal with breast cancer is admitted to the hospital, and an intravenous catheter is placed. Temperature-sensitive acoustic nanoparticles bearing a monoclonal antibody fragment directed against $\alpha_v\beta_3$ integrin on neovascular cells is administered at a dose of between 0.1 and 1.0 ml/kg body weight, preferably 0.25 to 0.5 ml/kg body weight. The agent is allowed to circulate and saturate the neovascular tissue receptors for between 15 minutes and 5 hours, preferably 1 to 2 hours. Baseline ultrasound images are obtained with a standard, commercially available ultrasound imaging device, such as those produced Agilent (Andover, MA), Acuson (Mountainview, CA), ATL (Bothel, WA), GE (Farifield, CT) Toshiba (Tokyo, JP), and similar devices. Sonothermography may be instituted with a

SONOTHERM 1000 Therapy system (Labthermics Technologies, Champaign, IL) or a related device according to the manufactures recommendations for the anticipated location of the suspected mass. The SONOTHERM 1000 is cycled to provide intermittent elevations (lasting less than 10 seconds each) in tissue temperature (between 42.° C and 45°C) in the selected region. The differential changes of acoustic contrast of the tumor vasculature targeted with nanoparticles and the surrounding normal tissue are used to specifically detect, localize and define the morphology of the tumor burden. These results are programmed into the SONOTHERM 1000 to refine the location and distribution for the hyperthermic radiation to be imparted. Throughout the subsequent hyperthermia therapy session, the temperature-dependent changes in acoustic backscatter imparted by the targeted nanoparticles within each region of the target tissue may be used to noninvasively determine deep tumor temperature. This information allows fine and continuous regulation of the hyperthermia protocol to closest possible tolerance, minimizing collateral damage to normal tissues and enhancing the overall safety of the procedure

EXAMPLE 8

This example illustrates methodology and instrumentation that can be used in applying the present invention to catheter-directed invasive hyperthermia.

The targeted temperature-dependent nanoparticles can be used in a catheter-based system as follows. A variety of therapeutic ultrasound catheters have been developed that allow highly focused heat generation for therapy and surgical application. An example of such a device is the multielement array system described by Lee et al. (*IEEE transactions of Biomedical Engineering. 4*:880-90, 1999). This unit has been demonstrated both *in vitro* and *in vivo* to achieve a therapeutic temperature rise (above 5° C) over 92% of a target volume of 30 mm x 30 mm x 35 mm. This and similar devices provide exquisite control of temperature distribution. This hyperthermia catheter system could be coupled with commercially available, intravascular ultrasound transducer technology to provide both fine detailed ultrasonic imaging of heated tissues at frequencies ranging from 10 to 50 MHz. These dual

imaging/therapeutic catheters could be utilized for both endoscopic an intravascular applications.

The catheter for hyperthermia and ultrasound system could be used in treating cancer as in the following example. A patient with suspected pancreatic cancer, would be admitted to the hospital, and an intravenous catheter placed. Temperature-sensitive acoustic nanoparticles bearing a monoclonal antibody fragment directed against $\alpha_v\beta_3$ integrin on neovascular cells is administered at a dose of between 0.1 and 1.0 ml/kg body weight, preferably 0.25 to 0.5 ml/kg. The agent is allowed to circulate and saturate the neovascular tissue receptors for between 15 minutes and 5 hours, preferably 1 to 2 hours. A combinational therapeutic/imaging ultrasonic catheter is advanced and images of the pancreas from a transgastric/transduodenal approach are obtained. The diagnosis, location and extend of the pancreatic tumor is confirmed through a temperature-dependent imaging protocol as previously described. The tumor is insonified to induce localized hyperthermia. The temperature of targeted tissue is monitored continuously by the changes in acoustic backscatter. Incremental temperature differences are color-mapped onto ultrasonic image displays that are repetitively updated and reviewed by the operator. The operator manually or the equipment automatically adjusts intensity or frequency of the ultrasonic beam to optimize tumor destruction and minimize collateral damage to normal tissues.

In view of the above, it will be seen that the several advantages of the invention are achieved and other advantageous results attained.

As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above description be interpreted as illustrative and not in a limiting sense.

All references cited in this specification are hereby incorporated by reference. The discussion of the references herein is intended merely to summarize the assertions made by their authors and no admission is made that any reference constitutes prior art. Applicant reserves the right to challenge the accuracy and pertinency of the cited references.

WHAT IS CLAIMED IS:

1. A method for changing acoustic reflectivity of an ultrasound target, the method comprising (1) administering to the target, a nongaseous acoustic imaging substance which binds to the target and produces a change in acoustic reflectivity with a change in temperature and (2) changing the temperature to produce a measurable change in acoustic reflectivity of the nongaseous acoustic imaging substance bound to the target.

2. The method according to claim 1 wherein the nongaseous acoustic imaging substance comprises an emulsion which contains a liquid fluorocarbon.

3. The method according to claim 5 wherein the fluorocarbon is perfluorooctane.

4. The method according to claim 1 wherein the nongaseous acoustic imaging substance comprises a ligand which binds to the target.

5. The method according to claim 4 wherein the ligand is an antibody, a fragment of an antibody, a polypeptide, a peptidomimetic, a polysaccharide, an aptamer, a lipid, a nucleic acid or a lectin.

6. The method according to claim 5 wherein the nongaseous acoustic imaging substance comprises a ligand conjugated with a biotin agent, an emulsion conjugated with a biotin agent and an avidan agent immobilized to the biotin-conjugated ligand and to the biotin-conjugated emulsion.

7. The method according to claim 5 wherein the nongaseous imaging substance comprises a ligand imobilized to a primer substance which is imobilized to an emulsion.

8. The method according to claim 1 wherein the emulsion further comprises a biologically active agent.

9. The method according to claim 8 wherein the biologically active agent is an antineoplastic agent, a radiopharmaceutical, a hormone, an analgesic agent, a nonsteroidal anti-inflammatory agent, an anesthetic agent, a sedative, a neuromuscular blocker, an antimicrobial agent, an antiparasitic agent, an antiviral agent, an interferon, a nitrate, an acne preparation, an androgenic agent, an antidiabetic agent, an anti-gout agent, an antihistamine, an antitussive agent, a decongestant, an expectorant, an antiulcer agent, a laxative, an anticoagulant, an immunization agent, an anticonvulsant agent, an anti-parkinsonian agent, an estrogenic agent, a thyroid agent, or an iron-containing anti-anemia agent.

10. The method according to claim 1 wherein the nongaseous acoustic imaging substance further comprises a magnetic resonance imaging substance, an electron spin resonance imaging substance, a spectroscopic imaging substance, a positron emission tomography imaging substance, an optical imaging substance, an x-ray imaging substance, a nuclear medicine imaging substance or a combination thereof.

11 The method according to claim 1 wherein the spectroscopic imaging substance comprises a nuclear magnetic resonance spectroscopic imaging substance or a raman spectroscopy imaging substance.

12. The method according to claim 11 wherein the nongaseous imaging agent comprises a paramagnetic or superparamagnetic element, a radioactive nuclide, or a photoactive agent.

13. The method according to claim 1 wherein changing the temperature comprises energizing the bound substance to increase temperature of the bound substance and enhance acoustic reflectivity of the surface.

14. The method according to claim 13 wherein the energizing is performed by generating energy from ultrasound, shortwave, microwave, magnetic radiation, electromagnetic energy or a combination thereof.

15. The method according to claim 1 wherein changing the temperature of the bound substance comprises reducing the temperature of the bound substance to produce a measurable decrease in acoustic reflectivity of the target.

16. The method according to 15 wherein reducing the temperature of the bound substance is performed as part of cryotherapy or heart bypass surgery.

17. The method according to claim 1 wherein changing the temperature comprises changing the temperature of the bound substance by at least 5°C.

18. A method for measuring enhanced acoustic reflectivity of an ultrasound target, the method comprising (1) administering to the target, a nongaseous acoustic imaging substance which binds to the target and produces a change in acoustic reflectivity with a change in temperature and (2) changing the temperature to produce a measurable change in acoustic reflectivity of the nongaseous acoustic imaging substance bound to the target, and (3) detecting change in acoustic reflectivity of the bound substance.

19. The method according to claim 18 wherein detecting comprises (a) measuring reflectivity prior to changing the temperature of the bound substance; (b) measuring reflectivity after changing the temperature of the bound substance; and (c) determining the change in reflectivity after changing the temperature of the bound substance compared to reflectivity prior to changing the temperature of the bound substance.

20. The method according to claim 19 wherein the nongaseous acoustic imaging substance comprises an emulsion which contains a liquid fluorocarbon.

21. The method according to claim 20 wherein the fluorocarbon is perfluorooctane.

22. The method according to claim 19 wherein the nongaseous acoustic imaging substance comprises a ligand which binds to the target.

23. The method according to claim 22 wherein the ligand is an antibody, a fragment of an antibody, a polypeptide, a peptidomimetic, a polysaccharide, an aptamer, a lipid, a nucleic acid or a lectin.

24. The method according to claim 23 wherein the nongaseous acoustic imaging substance comprises a ligand conjugated with a biotin agent, an emulsion conjugated with a biotin agent and an avidan agent immobilized to the biotin-conjugated ligand and to the biotin-conjugated emulsion.

25. The method according to claim 23 wherein the nongaseous imaging substance comprises a ligand immobilized to a primer substance which is immobilized to an emulsion.

26. The method according to claim 19 wherein the emulsion further comprises a biologically active agent.

27. The method according to claim 26 wherein the biologically active agent is an antineoplastic agent, a radiopharmaceutical, a hormone, an analgesic agent, a nonsteroidal anti-inflammatory agent, an anesthetic agent, a sedative, a neuromuscular blocker, an antimicrobial agent, an antiparasitic agent, an antiviral agent, an interferon, a nitrate, an acne preparation, an androgenic agent, an antidiabetic agent, an anti-gout agent, an antihistamine, an antitussive agent, a decongestant, an expectorant, an antiulcer agent, a laxative, an anticoagulant, an immunization agent, an anticonvulsant agent, an anti-parkinsonian agent, an estrogenic agent, a thyroid agent, or an iron-containing anti-anemia agent.

28. The method according to claim 19 wherein the nongaseous acoustic imaging substance further comprises a magnetic resonance imaging substance, an electron spin resonance imaging substance, a spectroscopic imaging substance, a positron emission tomography imaging substance, an optical imaging substance, an x-ray imaging substance, a nuclear medicine imaging substance or a combination thereof.

29. The method according to claim 19 wherein the spectroscopic imaging substance comprises a nuclear magnetic resonance spectroscopic imaging substance or a raman spectroscopy imaging substance.

30. The method according to claim 29 wherein the nongaseous imaging agent comprises a paramagnetic or superparamagnetic element, a radioactive nuclide, or a photoactive agent.

31. The method according to claim 19 wherein changing the temperature comprises energizing the bound substance to increase temperature of the bound substance and enhance acoustic reflectivity of the surface.

32. The method according to claim 31 wherein the energizing is performed by generating energy from ultrasound, shortwave, microwave, magnetic radiation, electromagnetic energy or a combination thereof.

33. The method according to claim 19 wherein changing the temperature of the bound substance comprises reducing the temperature of the bound substance to produce a measurable decrease in acoustic reflectivity of the target.

34. The method according to 33 wherein reducing the temperature of the bound substance is performed as part of cryotherapy or heart bypass surgery.

35. The method according to claim 19 wherein changing the temperature comprises changing the temperature of the bound substance by at least 5°C.

36. A method for monitoring temperature of a tissue in a patient, the method comprising (1) administering to the patient, a nongaseous acoustic imaging substance which binds to the tissue and changes acoustic reflectivity with changes in temperature, (2) detecting acoustic reflectivity of the nongaseous acoustic imaging substance bound to the tissue (3) calculating temperature of the nongaseous acoustic imaging substance bound to the tissue.

37. The method according to claim 36 wherein the method monitors a change in temperature, wherein the method further comprises changing the temperature of the tissue and the nongaseous acoustic imaging substance bound to the tissue, and wherein detecting comprises detecting the change in acoustic reflectivity of the nongaseous acoustic imaging substance bound to the tissue.

38. The method according to claim 36 wherein the nongaseous acoustic imaging substance comprises an emulsion which contains a liquid fluorocarbon.

39. The method according to claim 38 wherein the fluorocarbon is perfluorooctane.

40. The method according to claim 36 wherein the nongaseous acoustic imaging substance comprises a ligand which binds to the tissue.

41. The method according to claim 40 wherein the ligand is an antibody, a fragment of an antibody, a polypeptide, a peptidomimetic, a polysaccharide, an aptamer, a lipid, a nucleic acid or a lectin.

42. The method according to claim 41 wherein the nongaseous acoustic imaging substance comprises a ligand conjugated with a biotin agent, an emulsion conjugated with a biotin agent and an avidan agent immobilized to the biotin-conjugated ligand and to the biotin-conjugated emulsion.

43. The method according to claim 41 wherein the nongaseous imaging substance comprises a ligand immobilized to a primer substance which is immobilized to an emulsion.

44. The method according to claim 36 wherein the emulsion further comprises a biologically active agent.

45. The method according to claim 44 wherein the biologically active agent is an antineoplastic agent, a radiopharmaceutical, a hormone, an analgesic agent, a nonsteroidal anti-inflammatory agent, an anesthetic agent, a sedative, a neuromuscular blocker, an antimicrobial agent, an antiparasitic agent, an antiviral agent, an interferon, a nitrate, an acne preparation, an androgenic agent, an antidiabetic agent, an anti-gout agent, an antihistamine, an antitussive agent, a decongestant, an expectorant, an antiulcer agent, a laxative, an anticoagulant, an immunization agent, an anticonvulsant agent, an anti-parkinsonian agent, an estrogenic agent, a thyroid agent, or an iron-containing anti-anemia agent.

46. The method according to claim 36 wherein the nongaseous acoustic imaging substance further comprises a magnetic resonance imaging substance, an electron spin resonance imaging substance, a spectroscopic imaging substance, a positron emission tomography imaging substance, an optical imaging substance, an x-ray imaging substance, a nuclear medicine imaging substance or a combination thereof.

47. The method according to claim 46 wherein the spectroscopic imaging substance comprises a nuclear magnetic resonance spectroscopic imaging substance or a raman spectroscopy imaging substance.

48. The method according to claim 47 wherein the nongaseous acoustis imaging agent comprises a paramagnetic or superparamagnetic element, a radioactive nuclide, or a photoactive agent.

49. The method according to claim 36 wherein changing the temperature comprises energizing the bound substance to increase temperature of the bound substance and enhance acoustic reflectivity of the surface.

50. The method according to claim 49 wherein the energizing is performed by generating energy from ultrasound, shortwave, microwave, magnetic radiation, electromagnetic energy or a combination thereof.

51. The method according to claim 36 wherein changing the temperature of the bound substance comprises reducing the temperature of the bound substance to produce a measurable decrease in acoustic reflectivity of the target.

52. The method according to 51 wherein reducing the temperature of the bound substance is performed as part of cryotherapy or heart bypass surgery.

53. The method according to claim 36 wherein changing the temperature comprises changing the temperature of the bound substance by at least 5°C.

54. A device for measuring changes in temperature of a target having a temperature-sensitive acoustic imaging substance bound thereto, the device comprising a component configured to change the temperature of the acoustic imaging substance, an ultrasound source configured to transmit acoustic energy to the target, an ultrasound detecting component configured to measure acoustic reflectivity of the surface and a comparator which determines acoustic reflectivity of the target upon changing temperature relative to acoustic reflectivity of the target in absence of changing temperature.

55. The device according to claim 54 wherein the comparator determines difference in acoustic reflectivity of the target prior to and after changing temperature of the acoustic imaging substance bound to the target.

56. The device according to claim 55 wherein the comparator determines the difference in acoustic reflectivity of the target upon changing temperature of the acoustic imaging substance bound to the target, compared to acoustic reflectivity of the target after the changed temperature of the acoustic imaging substance bound to the target is diminished.

57. The device according to claim 54 wherein the ultrasound source and the ultrasound imaging component comprise at least one ultrasonic transducer.

58. The device according to claim 54 wherein the component configured to change the temperature of the acoustic imaging substance comprises an energy source.

59. The device according to claim 58 wherein the eneregy source produces ultrasound, shortwave, microwave, magnetic radiation, electromagnetic energy or a combination thereof.

60. The device according to claim 59 wherein the energy source is an ultrasound energy source having an intensity of greater than 0.1 W/cm^2 and less than 2000 W/cm^2.

61. The device according to claim 54 wherein the component configured to change the temperature of the acoustic imaging substance is an energy absorber.

62. The device according to claim 61 wherein the energy absorber comprises a cryogenic probe.

63. The device according to claim 54 wherein the component confugured to change the temperature of the acoustic imaging substance is configured to change the temperature of the bound substance by at least 5°C.

64. The device according to claim 54 wherein the comparator is configured to produce an image comprising the difference in acoustic reflectivity of the surface prior to and after increasing temperature of the acoustic imaging substance.

65. The device according to claim 64 wherein the comparator is configured to produce a colorized image comprising the difference in acoustic reflectivity of the surface prior to and after increasing temperature of the acoustic imaging substance.

66. The device according to claim 54 further comprising a component configured to perform magnetic resonance imaging, electron spin resonance imaging , spectroscopic imaging, positron emission tomography imaging, optical imaging, x-ray imaging nuclear medicine imaging or a combination thereof.

67. The method according to claim 66 wherein the spectroscopic imaging comprises nuclear magnetic resonance spectroscopic imaging or a raman spectroscopy imaging.

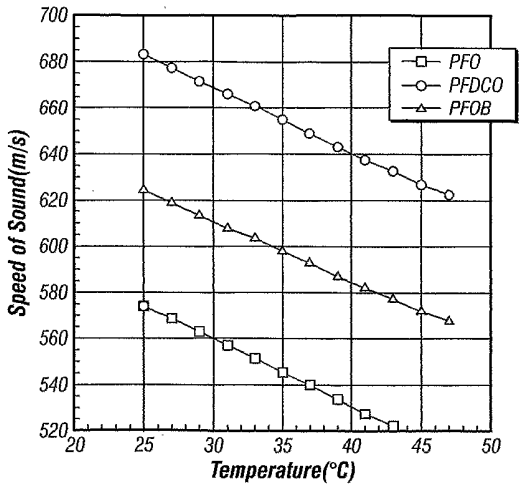
700
680
660
640
620
600
580
560
540
520
20
25
30
35
40
45
50
Speed of Sound(m/s)
Temperature(°C)
PFO
PFDCO
PFOB

FIG. 1

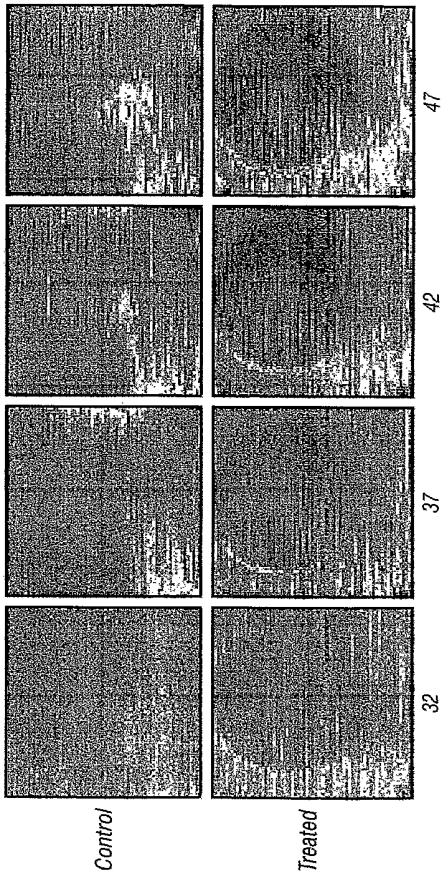

FIG. 2

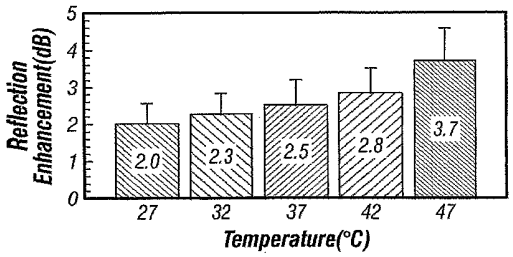
Reflection Enhancement(dB)
5
4
3
2
1
0
2.0
2.3
2.5
2.8
3.7
27
32
37
42
47
Temperature(°C)

FIG. 3

Before After

FIG. 4

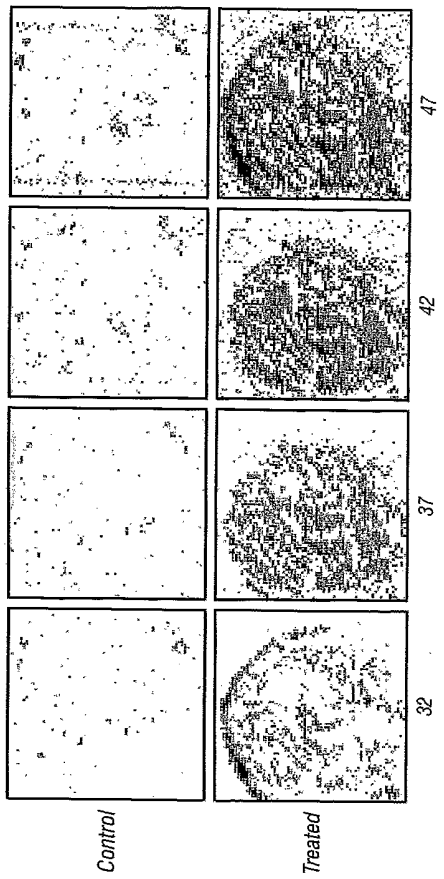

FIG. 5

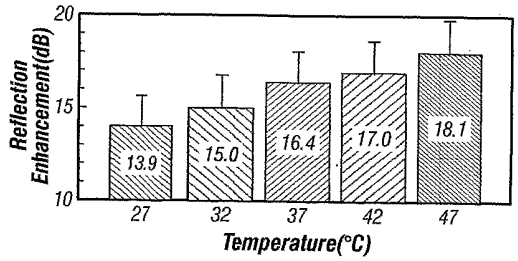
Reflection Enhancement(dB)
20
15
10
13.9
15.0
16.4
17.0
18.1
27
32
37
42
47
Temperature(°C)

FIG. 6

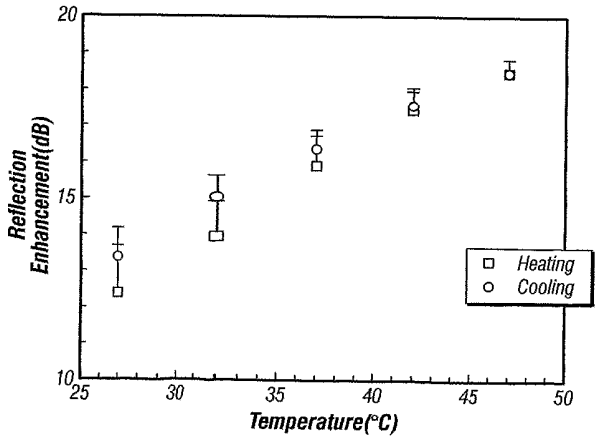
Reflection Enhancement(dB)
20
15
10
25
30
35
40
45
50
Heating
Cooling
Temperature(°C)

FIG. 7

【国際調査報告】

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/02651

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :A61B 8/00, 5/05, 5/055, 5/06; A61K 9/127, 9/133, 9/14, 9/107
US CL :424/9.5, 9.51, 9.52; 600/407, 436-442, 458; 606/3; 73/587
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 530/300, 350, 351; 514/2, 8, 12, 885; 424/85.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EAST, CAPlus
search terms: ultrasound, biotin, avidin, acoustic, photoactive, optoacustic

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,612,057 A (LANZA et al.) 18 March 1997, col. 9-11.	1-53
Y	US 5,989,520 A (LANZA et al.) 23 November 1999, abstract, col. 14, lines 14-67; col. 15, lines 1-44; col. 25, lines 1-67; col. 27-28.	1-67
Y	US 6,159,445 A (KLAVENESS et al.) 12 December 2000, abstract, col. 28, lines 36-67; col. 29-30.	1-53
Y	US 6,123,923 A (UNGER et al.) 26 September 2000, abstract, col. 100-104; col. 121, lines 18-39.	1-53
Y	US 5,840,023 A (ORAEVSKY et al.) 24 November 1998, abstract, cols. 5, 7-8.	54-67

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 10 SEPTEMBER 2001	Date of mailing of the international search report 18 DEC 2002
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/08651

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,977,538 A (UNGER et al.) 02 November 1999, abstract, col. 5, 6-8.	54-67

Form PCT/ISA/210 (continuation of second sheet) (July 1998)★

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Ｆターム(参考) 4C085 HH03 HH05 HH07 HH09 JJ03 KB39 KB87 KB95 KB99 LL01 LL07
4C096 AA18 FC14 FC20
4C601 DD22 DE06 DE07 FF13

专利名称(译)	用温度相关对比剂增强超声检测		
公开(公告)号	JP2005503836A	公开(公告)日	2005-02-10
申请号	JP2002560714	申请日	2002-01-30
[标]申请(专利权)人(译)	巴内斯 - 朱威胥医院		
申请(专利权)人(译)	巴恩斯 - 犹太医院		
[标]发明人	ランザグレゴリーエム ウィクラインサムエルエイ ホールクリストファーエス		
发明人	ランザ、グレゴリー、エム ウィクライン、サムエル、エイ. ホール、クリストファー、エス.		
IPC分类号	G01R33/28 A61B5/055 A61B8/00 A61K9/133 A61K49/00 A61K49/04 A61K49/22 A61K51/00		
CPC分类号	A61K49/223 A61K49/226 Y10S514/937 Y10S514/938		
FI分类号	A61B8/00 A61K49/00.A A61K49/00.C A61K49/04.A A61B5/05.383 G01N24/02.B A61B5/05.400 A61K49/02.A		
F-TERM分类号	4C085/HH03 4C085/HH05 4C085/HH07 4C085/HH09 4C085/JJ03 4C085/KB39 4C085/KB87 4C085/KB95 4C085/KB99 4C085/LL01 4C085/LL07 4C096/AA18 4C096/FC14 4C096/FC20 4C601/DD22 4C601/DE06 4C601/DE07 4C601/FF13		
优先权	09/774278 2001-01-30 US		
其他公开文献	JP2005503836A5 JP4202139B2		
外部链接	Espacenet		

摘要(译)

公开了用于基于与超声目标结合的温度依赖性造影剂的温度和超声反射率的变化来增强超声检测的方法和装置。所述方法和装置可单独用于增强成像或与药物递送结合使用，具有治疗方法，例如热疗或冷冻疗法或其他成像方式。