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(21) 出願番号	特願2002-522862 (P2002-522862)	(71) 出願人	390040420
(86) (22) 出願日	平成13年8月28日 (2001.8.28)		マックス・プランク・ゲゼルシャフト・ツ
(85) 翻訳文提出日	平成15年2月28日 (2003.2.28)		ア・フェルデルング・デア・ヴィッセンシ
(86) 国際出願番号	PCT/EP2001/009908		ャフテン・エー・ファオ
(87) 国際公開番号	W02002/017888		ドイツ連邦共和国 ベルリン (番地なし)
(87) 国際公開日	平成14年3月7日 (2002.3.7)		B e r l i n , B R D
(31) 優先権主張番号	00118615.4	(74) 代理人	100061815
(32) 優先日	平成12年8月28日 (2000.8.28)		弁理士 矢野 敏雄
(33) 優先権主張国	欧州特許庁 (EP)	(74) 代理人	100094798
(31) 優先権主張番号	01112600.0		弁理士 山崎 利臣
(32) 優先日	平成13年5月23日 (2001.5.23)	(74) 代理人	100099483
(33) 優先権主張国	欧州特許庁 (EP)		弁理士 久野 琢也
(81) 指定国	EP (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), CA, JP, US		

最終頁に続く

(54) 【発明の名称】 高分子電解質多層カプセルの制御された持続性の放出特性

(57) 【要約】

反対電荷を有する高分子電解質の L b L アセンブリ法をフルオレセイン粒子の被覆のために適用した。4 ~ 9 μm を有するこれらの粒子は pH 2 でフルオレセインの堆積により製造した。ポリスチレンスルホネート (PSS) およびポリアリルアミン (PAH) を使用して高分子電解質シェルをフルオレセインコア上に作製した。高分子電解質シェルを通過するコアの溶解によるフルオレセイン分子の放出は、蛍光強度の増大により pH 8 で観察された。蛍光放出を維持するために十分な高分子電解質層の数は、8 ~ 10 であることが判明した。引き続き吸収された層はコア溶解時間を数分間延長した。厚さ 20 nm の高分子電解質多層の透過率は約 10^{-8} m / s であった。蛍光拡散のメカニズムおよび浸透圧により支持される放出を論じる。放出プロファイルおよび封入された材料のための放出特性を制御するためにシェル形成のための L b L 法の可能な適用の概要を示す。pH、温度および塩濃度の周囲条件もまた高分子電解質多層カプセルの透過性を制御するために変更した。

【特許請求の範囲】

【請求項 1】

高分子電解質多層カプセルの透過性を制御するための方法において、pH、温度、光、塩濃度、イオン組成、イオン濃度、イオン強度、溶剤組成または溶剤濃度のパラメータの少なくとも1つを、カプセルの製造、貯蔵および／または使用の間に調節および／または変更することを特徴とする、高分子電解質多層カプセルの透過性の制御方法。

【請求項 2】

高分子電解質多層カプセルの透過性を制御するための方法において、所望の透過性が得られるように高分子電解質層の数および／または化学組成、疎水性、極性、剛性、分子量、層成分、特に高分子電解質の電荷もしくは電荷密度を調節することを特徴とする、高分子電解質多層カプセルの透過性の制御方法。

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【請求項 3】

高分子電解質多層カプセルを不透過性にするために少なくとも8のpHを調節する、請求項1記載の方法。

【請求項 4】

高分子電解質多層カプセルを透過性にするために6より低いpHを調節する、請求項1記載の方法。

【請求項 5】

少なくとも 2×10^{-2} Mの塩濃度を調節する、請求項1から4までのいずれか1項記載の方法。

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【請求項 6】

5×10^{-3} Mより低い塩濃度を調節する、請求項1から4までのいずれか1項記載の方法。

【請求項 7】

温度を少なくとも50℃に設定する、請求項1から6までのいずれか1項記載の方法。

【請求項 8】

温度を50℃よりも低く設定する、請求項1から7までのいずれか1項記載の方法。

【請求項 9】

アルコールおよび／または水を含有する溶剤組成物を使用する、請求項1から8までのいずれか1項記載の方法。

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【請求項 10】

高分子電解質層の数が少なくとも8である、請求項2記載の方法。

【請求項 11】

リピド、界面活性剤、染料、薬剤分子、ナノ粒子および／または生体高分子、たとえば多糖類、ポリペプチド、核酸を層成分として含む、請求項1から10までのいずれか1項記載の方法。

【発明の詳細な説明】

【0001】

本発明は薬剤をカプセル化および放出するためのマイクロコンテナ (micro container) の透過性を制御する方法、特に高分子電解質多層カプセルの持続放出性の制御に関する。

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【0002】

先進的な医薬調製物の開発における重要な課題は、生理活性材料の持続放出を提供するデリバリーシステムを洗練することに取り組んでいる。多くの場合、これらのデリバリーシステムは $10^2 \sim 10^5$ nmの範囲の大きさのポリマー粒子からなる。薬剤分子はポリマーマトリックス中またはコア・シェル構造中に埋め込まれている。コア・シェル中での分解速度は生理活性コア材料の放出速度を決定する。

【0003】

原則として、薬剤粒子の表面上にポリマーまたは生体高分子を吸着させることにより [1]、またはモノマーを吸着させ、引き続き界面において重合することにより [2~4]、

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活性コアの周囲にシェルを製造することができる。シェルの組成はさらに特定の機能を提供することができる。これはコアと溶剤との相互作用を促進する、または特定の所望の化学的特性を付与するように調節してもよい。シェルは生理活性材料を含有するコアを方向付ける、または操作するために磁氣的、光学的、導電性または標的特性を有していてもよい。

【0004】

最近ではポリマーカプセルを構成する新しいタイプのシェル構造が導入された〔5～7〕。これらの新規の中空ポリマーカプセルはサブミクロンおよびミクロン範囲の規定のサイズと、調整可能な壁特性を有する〔7、13～15〕。これらのカプセルは、コロイド粒子上の高分子電解質のLbL(layer-by-layer)アセンブリに引き続きコロイドコアの除去により製造される。LbLアセンブリは反対電荷を有する種、たとえば高分子電解質をコロイド粒子の表面上に交互に吸着させることにより行う〔16、1〕。LbL吸着のための駆動力は入ってくるポリマーと表面との間の静電引力である。

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【0005】

約60nm～数十ミクロンの大きさの範囲の種々のコア、たとえば有機もしくは無機材料、生物細胞、薬剤結晶またはエマルション滴が、LbL技術により多層を形成するためのテンプレートとして使用されている。現在までに多種多様な異なった物質、たとえば合成および天然高分子電解質、生体高分子、タンパク質、核酸、磁性および蛍光無機ナノ粒子、リピドなどがコロイド粒子上に多層シェルを形成するための層成分として使用された。シェル壁の厚さはその製造の条件に依存する。ナノメートル範囲で調整することもできる。コロイド粒子上の多層被膜の厚さはたとえば層の数を変更する吸着によりナノメートル範囲で調整することができる。カプセル壁が半透過性の特性を有することが確立された〔8、9〕。これらは小さい分子、たとえば染料およびイオンに対しては透過性であるが、その一方で高分子量を有する化合物を排除する〔17、9〕。

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【0006】

多種多様な材料が存在し、これらのカプセル化は異なった技術分野での適用のため、たとえば触媒、化粧品、医薬、バイオテクノロジー、栄養学などのために所望されている。

【0007】

カプセルにポリマーを充填するための可能なアプローチの1つは、カプセルを形成しながら所望のポリマーをシェル構造の内層に埋め込み、かつ高分子電解質、たとえば多価イオンを中空シェルの内層からその内部へと脱着することである〔18〕。次いでこれらのイオンを除去することによりポリマーがカプセル内部へと放出される。

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【0008】

もう1つのアプローチはカプセル壁の選択透過性を利用してカプセル内部でポリマーを直接合成することである〔19〕。カプセルはこれにより首尾よく充填される一方で、これらの方法はポリマーの種類に関して限定的な適用性を有するのみであり、かつコアの溶解または重合のためにしばしば厳しい条件、たとえば低いpH、コアの分解のために使用される酸化剤または有機溶剤、または合成の間に高めた温度を使用しなくてはならない。

【0009】

制御可能な、または調節可能な充填ならびに放出特性を有するシステムを提供することが所望されていた。特にカプセル壁の透過性を変更することにより、材料をカプセルへ充填する、またはカプセルから放出することを可能にする方法が所望されていた。さらに、多くの適用にとってカプセル充填ならびに特殊な環境条件下でのその後の放出のプロセスを制御するために、定義され、かつ制御可能なカプセル壁の透過性が要求される。充填は有利に迅速であるが、しかし放出は多くの適用にとって緩慢であるという事実に基づいて、透過性が切り替え可能であることがさらに所望される。

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【0010】

従ってカプセル壁の特性に影響を与え、該特性を変更もしくは切り替えるための方法を提供することが本発明の課題であった。本発明のもう1つの課題は高分子をカプセル内に導入し、かつ該分子に対するカプセルの透過性を切り替え、かつ制御するための手段を提供

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することであった。

【0011】

これらの課題は、カプセルの製造および使用の間の反応条件を変更することによって高分子電解質多層カプセルの透過性を制御するための方法において、pH、温度、塩濃度、イオン組成、イオン濃度、イオン強度、溶剤組成または溶剤濃度の反応条件の少なくとも1つを変更することを特徴とする方法により解決される。

【0012】

本発明はさらに高分子電解質多層カプセルの透過性を制御するための方法に関し、その際、所望の透過性が得られるように高分子電解質層の数を調節する。

【0013】

本発明によれば高分子電解質多層カプセルの透過性は、カプセルの環境のパラメータ、たとえばカプセルを製造または使用する間の反応条件、カプセルがその中に含有されている媒体またはカプセルがその中へと輸送される媒体により決定、変更および／または制御することができる。高分子量の化合物に対する透過性も低分子量の化合物に対する透過性も、異なった適用における要求に応じて調節できることが判明した。

【0014】

ここで使用される高分子量の化合物または高分子とは、少なくとも30000Da、より有利には少なくとも50000Daおよび最も有利には少なくとも70000Daの分子量を有する分子である。低分子量の化合物または小さい低分子とは10000Daよりも小さい、有利には5000より小さい、および最も有利には1000Daよりも小さい分子量を有する分子である。

【0015】

本発明による透過性の制御は、カプセル内に分子を封入し、かつ該分子を規定の方法で、たとえば延長された期間にわたって、または所望の規定の場所もしくは時点で放出するための独自の手段を提供する。

【0016】

本発明による方法によって、特にカプセルの透過性を可逆的に修正することが可能になる。このことによってカプセルの環境条件を修正することにより簡単な方法で、特に所望の活性物質によってカプセルを充填する、または特にカプセル中に封入された活性物質を放出することがそれぞれ可能になる。従ってたとえば透過性を増大することにより、完成したカプセルを後から活性物質で充填することができる。活性物質がカプセル壁を透過することができるこのような「開放された条件(open condition)」は本発明によれば、カプセルの50%以上、より有利には70%以上、および最も有利には90%以上が透過性である場合に存在する。カプセルを充填した後でたとえば貯蔵または輸送する間に、適切な条件を調節することによって、封入された活性物質に対するカプセル壁の透過性を低減することができるので、活性物質はカプセルから放出されない。しかしカプセルのこのような「閉じられた条件(closed condition)」はさらに同時に、おそらく不所望の物質がカプセル内に入ることを防止する。カプセル壁の透過性を増大することによりそれぞれ所望の放出時間および放出場所で活性の薬剤を規定の方法で、たとえば遅延して放出することができる。さらに所望の組織中で優勢な条件下で、封入された活性物質に対して高い透過性を有するカプセル壁を使用することにより、細胞の特定のコンパートメントまたは有機体の特定の領域での放出を得ることが可能である。

【0017】

本発明により規定の方法で環境条件によってカプセル壁の透過性ひいては高分子の封入および排除を調整することができる。高分子のpH制御された取り込みおよび放出のために、その電荷がpHに依存する高分子電解質からなるカプセルを使用することができる。

【0018】

持続性の薬剤放出のための高分子電解質カプセルの実地での使用は、高分子電解質壁を通過する小さい分子の透過に関する定量的なデータを必要とし、そのデータをここに示す。

【0019】

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持続性放出の観点から、小さい極性分子を一度カプセル封入してしまえば該分子の層透過性を低減することができることが有利である。この目標に到達するための1つの可能な方法は、層成分としてリピドを使用することである〔9、10〕。その際、より厚いカプセル壁の形成は透過性を低減するために有利かつ容易な方法であると詳細に示されている。層の数を増加することによって、カプセル内に封入された材料または分子によるシェルの浸透が低減する。

【0020】

このアプローチを検査するためにフルオレセインマイクロ粒子を異なった数の高分子電解質層で被覆した。フルオレセインのような染料は多種の薬剤のためのモデル物質として考えることができる。その後、pH値の変更によりコアの溶解を開始し、かつバルク中の蛍光の増大により監視した。

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【0021】

低分子量の化合物からなるコアの周囲に配置された高分子電解質多層シェルは、コアが溶解する条件下での放出に対してバリア特性をもたらしことが判明した。この発見は、延長され、かつ制御される放出特性を有するシステムを製造するための新規のアプローチである。放出は配置される高分子電解質層の数によって調節することができる。低分子量の化合物に対するカプセル透過性はカプセル中の高分子電解質層の数に著しく依存することが判明した。

【0022】

異なった特性を有する多種多様な合成高分子電解質、リピドおよび多糖類がすでに多層アセンブリのために使用されてきた〔9、12〕。このことによってシェルの放出特性を調整すると共に、種々のコアを使用する生物学的親和性および可能性を保証する多数の可能性が提供される。LBL技術によるシェルのアセンブリは、物質の制御され、かつ延長された放出が必要とされるバイオテクノロジーの適用のための新たな経路を開く。薬剤の調製、放出およびデリバリー、有機体中での濃度の制御およびその吸収の周期性に関する数多くの問題を、堆積物およびナノ結晶上のシェルの形成により解決することができる。さらに高分子電解質層に標的特性を与えることは困難でない。この方法で特定の、もしくは損傷した組織に対する、高分子電解質多層被覆された薬剤の親和性を増大することができる。

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【0023】

以下ではモデル物質としてフルオレセイン（分子量～350Da）を使用して、より定量的な条件でカプセル壁を形成している層の数と関連させてカプセルの放出特性を論じる。

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【0024】

フルオレセインのような分子のシェルの透過はその透過率（P）により記載される。式（1）はバルク中のフラックス（J）とシステムのパラメータおよびフルオレセイン濃度の変化の速度 C_e を結びつける。この速度が一定の場合、蛍光の増加の傾斜から透過率（P）を容易に計算することができる。（図3のパート2）。

【0025】

【数1】

$$\frac{dc^e}{dt} V_0 = J = P \cdot (c^i - c^e) \cdot S \quad (1)$$

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【0026】

V_0 が溶液の体積である場合、Jは全表面積Sを有するカプセル壁を通過するフルオレセインフラックスであり、かつ $(C^i - C^e)$ はカプセルの内側の (C^i) および外側の (C^e) のフルオレセイン濃度の差である。

【0027】

固体のフルオレセインのコアがカプセル内に存在する限り、カプセルの内側は25mg／

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m l の飽和のフルオレセイン溶液 C^s を含有する。従って式 1 の右側のプロセスの開始時点での濃度差は $C^i = C^s$ によって問題なく換算することができる。カプセルは平均直径 $5 \mu m$ を有する球形であると想定された。従って透過率は

【0028】

【数2】

$$P = \frac{dc/dt \cdot V_0}{c^s \cdot S} \quad (2)$$

【0029】

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から計算することができる。

【0030】

8～18層に関して透過率の値は $10^{-8} m/s$ のオーダーである。1つの高分子電解質層の厚さが $2 nm$ であることを想定して、透過率は透過率とシェル壁の厚さとをかけ算することによって拡散係数 (D) へと換算することができる。計算される拡散係数は $10^{-15} m^2/s$ のオーダーである。

【0031】

高分子電解質多層の透過性が絡み合ったポリマーの網状構造を通過する網状構造により生じる場合、これは層の厚さの逆数で調整すべきである。層の数の相関としての厚さ×透過率の挙動は図4に示されている。見て分かるように透過率は層の数が増えると共に、直線的な厚さの増大から予測されるよりもはるかに早く低減する。およそ8層から透過率とシェルの厚さの商は一定となり、その際、透過率がいまや厚さの増大によって制御できる、たとえば拡散を限定する領域が高分子電解質層であることを示す。この発見は、最初の8層の構造がその後配置される層とは異なっていることを示す初期の観察 [11] と一致する。これらのより深い層はより緻密であり、その際、図4で推論することができるような予想拡散係数を五分の一に低減する。

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【0032】

透過係数はフルオレセインの放出曲線の第三段階における放出プロファイルからも計算することができる。この段階における放出の時間依存性が指数関数的であることが予測される：

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【0033】

【数3】

$$c^e(t) \sim \left(1 - e^{-\rho \cdot \frac{\bar{S}}{V} t} \right), \quad (3)$$

【0034】

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【外1】

$\bar{S}$ および V は平均的なカプセルの表面積および体積を示す。しかしこの式から予想される透過率は直線的な領域の傾斜から計算される透過率よりも 1 オーダー小さい。現在、この矛盾について可能な説明が 2 つ存在する。特徴的な時間放出 $V/\bar{S}P$ が粒子の半径と共に直線的に増大しているために、粒子の多分散性が見かけは延長された放出に貢献しているか、または放出の終了に向かう浸透圧の緩和が透過率を低減する。

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【0035】

以下では透過メカニズムをより詳細に議論する。水で満たされた気孔を通過する拡散と、高分子電解質多層シェルの均一な層を通過する「バルク」拡散メカニズムとを区別することができる。より薄い壁に関して、シェルの厚さへの著しい依存性は、さらなる層の堆積により連続的に閉鎖される気孔の存在によって、または厚さに依存する拡散係数によって説明することができる。後者は実際に、 $10^{-18} \sim 10^{-20} \text{ m}^2 / \text{sec}$ の典型的な D の値が得られる平坦な高分子電解質膜により調査される透過と共に観察された [11]。我々結果を先行の研究結果とを比較するために、次の相違点に言及する。

【0036】

— [11] に記載された方法によれば膜はそれぞれの吸着段階の後で乾燥することにより製造されたが、これは粒子を被覆する間には不可能であり、かつより緻密な膜につながる。

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【0037】

— 測定は染料試料を規定の厚さで膜中へ堆積させ、かつクエンチ剤 (quencher) の拡散に基づいた、時間に依存する蛍光の変化を観察することからなっていた。たしかにこのケースに該当する十分に小さい気孔濃度に関して、この技術は「バルク」拡散に対して感受性であるにすぎず、かつ気孔を通過する透過は反映しない。

【0038】

従って透過率データから得られる拡散係数は、[11] で測定された値よりも大きいことが予測される。それでも、3～4 オーダーの大きさの違いはこのことによって説明することができず、従ってこれらはむしろ気孔を通過する拡散に相応する可能性が高い。拡散係数 D と、気孔容積中での拡散に関する上限であるバルク水中での拡散係数 D ($\sim 10^{-10} \text{ m}^2 / \text{s}$) を比較すると、壁内部の気孔容積のフラクションは 10^{-5} よりも大きいことが予測される。

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【0039】

次に気孔形成の可能なメカニズムについて扱う。壁の不安定化が予測されうる。というのも、 pH 値が 8 に向かって上昇すると、 PAH のアミノ基が脱プロトン化され、かつこのことによって多層中に気孔が形成される。気孔形成のもう 1 つの可能性は、フルオレセインコアの溶解の結果として、内部に浸入する水による浸透圧である。関連する静水圧の差は、壁中に圧力を生じ、この圧力は存在する気孔を拡大するか、または新たな気孔を形成する。図 3 (パート 1) において見られるように、特に層のより大きな番号に関して、溶解の開始時に放出は持続性である。内側から浸透圧がかかる高分子電解質シェルは、フルオレセイン分子がカプセルを出るための経路を作るまでフルオレセインの放出に抵抗することが予測される。気孔は高分子電解質多層のこの再配置の結果として形成される。テンプレートおよび得られるカプセルが異なった直径を有し、かつ不均一な壁厚を有することに注意すべきである。従って拡散係数は平均的であるが、しかしより遅い放出プロファイルを有するより大きなテンプレートがなお存在していてもよい。

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【0040】

まとめると、高分子電解質カプセル壁の層の数を増大することによってカプセルの透過率が低下しうることが認められる。遅延された放出のために有利には 8 以上、より有利には

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9以上、および最も有利には10以上の層を有するカプセルを使用する。

【0041】

本発明は有利な実施態様では、材料および分子、たとえば高分子、生体高分子、薬剤などを予め形成された中空の高分子電解質カプセル中に封入するための新規の方法に関する。本発明のこの実施態様によれば、材料／分子の周囲にカプセルを形成するよりもむしろ、材料および／または分子を中空カプセル中へ導入することができる。

【0042】

高分子電解質多層の特性および構造は、周囲の媒体の種々の物理的および化学的条件に対して敏感であることが判明した。特にpHはその物理化学的なパラメータの1つであり、これは高分子電解質錯体間の状態〔20、21〕に、特に錯体中の1つの高分子電解質の電荷がpHに依存する場合に、影響を与える。高分子電解質対、ポリ（スチレンスルホネート）（PSS）および比較的弱いポリカチオンであるポリ（アリルアミンヒドロクロリド）（PAH）は、平坦なコロイド表面上に多層の膜を製造するために最もよく使用されており（1）、かつ本発明を記載するための高分子電解質対の例として使用する。しかし、本発明はこの特定の高分子電解質対に限定されない。

【0043】

異なった高分子電解質を使用することにより、透過率をさらに変更することができる。カチオンポリマーとアニオンポリマーとを組み合わせることによりほぼ無限の変更の可能性が生じる。特性値、たとえば極性およびポリマーの剛性は、化学組成により調節することができ、かつ異なった物質クラスに対して特殊な効果を有する。たとえば極性を低下させることにより、高い疎水性が得られ、このことにより極性の、水溶性物質に対する透過性が低下し、その一方で無極性の、油溶性物質に対する透過性は強化される。

【0044】

さらにカプセルの透過挙動は、使用される高分子電解質の分子量および分岐度により著しく修正することができる。分子量は有利には10000および500000g／モルの間で設定され、その際、一般により大きな分子量および分岐の増大はカプセルの安定性を高め、かつ透過率を低下させる。

【0045】

媒体のpH値を変更することによりマイクロカプセルの壁の透過率を変えることができる。pH誘発される透過率の変化の特殊なメカニズムに結びついていることは意図されていない一方で、pHが変化する際の高分子電解質の荷電の変化は気孔形成を誘発することができる（22）か、または高分子電解質の網状構造を緩め、従ってポリマーの浸透を可能にすることが想定される。pHの変化に付随するイオン濃度の影響は、透過率の変化にも貢献しうる。

【0046】

カプセル壁を開放および閉鎖の状態に切り替えるこの可能性は、分子、特にポリマー、生体高分子およびナノ粒子の取り込みおよび放出を制御するために便利で、効果的な手段である。たとえば低いpH値でカプセルを充填し、かつpHを上昇させた後に材料は内側に封入される。放出のためにふたたびpHをわずかに低下させ、その際、放出の動態はpHにより調整することができる。ここに示した、制御して高分子を高分子電解質カプセル中に充填し、かつ該カプセルから放出する可能性により、広い適用が可能となる。記載した、膜中の高分子電解質網状構造の、pH誘発される透過率の変化は高分子電解質多層の透過率を修正するための一般的なメカニズムであり、かつシェルの特定の組成に限定されない。弱い高分子電解質からなる平坦な高分子電解質膜に関する調査は、気孔がpH値または塩濃度の変更により生じることを示している。

【0047】

高分子電解質、特に弱い高分子電解質からなるカプセルの透過率はpH値に著しく依存することが判明した。カプセルは、カプセル壁を形成している2つの高分子電解質の電荷が代償されているpHで高分子に対して不透過性であるが、弱い高分子電解質が完全に荷電しているpHでカプセルは開放されている。この依存性はpHによりカプセルを開放およ

び閉鎖することによってポリマーをカプセル封入するための機会を与える。開放された状態でAFMにより100～300nmの大きさの範囲の気孔がカプセル壁中に観察される。pHの変化により気孔を形成するプロセスのもう1つの顕著な特徴は、気孔の形成または透過率の増大が可逆的なことである。

【0048】

反対にカプセルのさらなる透過性は該カプセルを構成している層の数に依存する。これらのファクターを両方使用することにより、低分子の化合物に対しても高分子の化合物に対してもカプセルの放出特性を制御する可能性が与えられる。酸性のpHで安定しているフルオレセイン粒子をコアとして使用することができる。該粒子を異なった数の高分子電解質層で被覆することができる。フルオレセインの溶解および放出の動力学を塩基性のpHで測定した。コアの分解時間はカプセル壁の厚さに著しく依存することが判明した。溶解の間、フルオレセインの持続性放出が観察された。

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【0049】

pHおよびカプセル壁の厚さによる透過率の制御は異なった物質をカプセル中に取り込み、かつカプセルから放出することを制御するための新たな方法として提案される。これは高分子電解質カプセルを多くの適用、たとえば薬剤担体、マイクロ反応器などのために使用するための新たな経路を開く。

【0050】

まとめると、pHを上昇させることによりカプセルの透過率を低下させることができ、かつpH値を低下させることによりカプセルの透過率を上昇させることができ、従って、このプロセスは可逆的であるといえることができる。カプセル壁は特に8より大きいpHで、より有利には10より大きいpHで不透過性（閉じられた状態）であり、その一方で6より小さいpHで、特に4より小さいpHでカプセル壁の透過性（開放された状態）が得られることが判明した。pHの変更は透過性を調整するための簡単な方法であり、かつ周囲の媒体を調節することによりカプセルのための透過性の切り替えを提供することが可能になる。

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【0051】

本出願は、塩濃度によりカプセル壁の透過性を制御することによって感受性の高分子を標的に合わせてカプセル封入し、かつ放出するための新規の、簡単かつ極めて緩和な方法を提供する。塩は生きている生物体において著しい重要性を有する。塩濃度は細胞質および細胞外媒体中で細胞膜の透過性、浸透圧およびエネルギーにより運転されるポンプによる能動的輸送によって著しく制御される。血漿中の塩化ナトリウムの生理学的濃度は約0.15Mであり、その他のイオン、たとえばカリウムおよびカルシウムは膜を横断する、および細胞中の信号変換にとって決定的である。

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【0052】

中空の高分子電解質カプセル、有利にはPAH/PSSから作られたカプセルの透過性は、 $0.5 \sim 2 \times 10^{-2} \text{ M/l}$ の狭い濃度範囲で塩濃度を変更することにより開放および閉鎖状態の間で可逆的に切り替えることができる。塩濃度の上昇は50000Daを上回る、有利には70000Daを上回る大きな分子に対するカプセル壁の可逆的な開放につながる。

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【0053】

塩濃度の上昇に伴う走査型電子顕微鏡画像における気孔の不在、比較的小さい透過率および壁中でのフェルスター共鳴エネルギー移動の段階的な低下は、高分子電解質間の静電相互作用およびその後のポリマーのより良好な拡散と関連した膨潤が弱くなることを示している。塩により誘発される透過性は、マイクロコンテナおよびナノコンテナ中の特に敏感な高分子、たとえば酵素、タンパク質またはDNAのカプセル封入および放出のための極めて簡単で、重要かつ効果的な方法である。

【0054】

まとめると、塩濃度を上昇させることによってカプセル壁の透過性を増大することができ、その一方で、塩濃度を低下させることにより透過性が低減する（閉鎖された状態）こと

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が認められる。有利にはカプセル壁は少なくとも 2×10^{-2} M、より有利には少なくとも 3×10^{-2} M および最も有利には少なくとも 1×10^{-1} M の塩濃度によって透過性になる。カプセルの閉鎖された状態が所望される場合、周囲の条件は最大で 5×10^{-3} M、より有利には最大で 1×10^{-3} M および最も有利には最大で 1×10^{-4} M の塩濃度に調節される。

【0055】

本発明により適切な塩は、少なくとも1つのアニオンおよび少なくとも1つのカチオンからなる全ての異極化合物である。アニオンおよびカチオンは1つ荷電した、もしくは複数荷電したイオンであってもよい。透過率を調節するために、有利には無機塩、好ましくは金属塩、たとえば金属ハロゲン化物、特にアルカリ金属ハロゲン化物を使用する。特に有利な塩の例はアルカリ金属カチオン、特に Li^+ 、 Na^+ 、 K^+ 、アルカリ土類金属カチオン、特に Mg^{2+} 、 Ca^{2+} または異なった金属カチオン、たとえば Ag^{3+} 、鉄イオンなど、およびアニオン、たとえばハロゲン化物のアニオン、または異なった無機アニオンを含有する塩である。金属はその他の正電荷を有する基、たとえばアンモニウム基、スルホニウム基またはホスホニウム基により交換されていてもよい。しかし有機塩、特に有機アニオンを含有する塩を使用することも可能である。

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【0056】

塩濃度以外に透過性は環境のイオン濃度およびイオン組成のそれぞれによっても調節することができ、その際、ここでもまたより高いイオン濃度に関して透過性が達成され、その一方で低いイオン濃度に関して不透過性のカプセル壁が得られる。

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【0057】

イオン組成は特に特定の役割を果たす。というのも、カプセルの透過性が周囲の媒体のイオンと、高分子電解質シェルの電荷との間の相互作用によって影響されるからである。周囲の媒体のイオン、たとえばカプセルシェルの高分子電解質と錯体を形成するイオンを適切に選択することにより、透過性を調節することができる。

【0058】

塩濃度、イオン組成およびイオン濃度への透過率の依存性は特に有利には、カプセル封入された活性物質を有機体、たとえば哺乳動物および特にヒトに投与することによって使用することができる。従ってカプセルはまず最初に、インビトロで上記の手段によってカプセル壁を活性物質に対して透過性にして、活性物質で充填することができる。次いでカプセル封入された活性物質を、たとえば貯蔵媒体の塩濃度を 5×10^{-3} M 未満に調節することによって安定した形で貯蔵することができる。これはたとえば蒸留水、有利には無菌の蒸留水中に貯蔵することによって行うことができる。充填したカプセルの投与は周囲の媒体の調整を伴い、その際、すでに記載したように、血漿中の塩化ナトリウムの生理的濃度は約 0.15 M である。この周囲の条件下でカプセルは活性物質に対して透過性であるので、活性物質が標的組織で放出される。

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【0059】

本発明は、高分子電解質カプセルが大きなポリマーに対して開放および閉鎖されるが、しかし小さい有機分子に対しても開放および閉鎖され、かつ温度処理により透過性を簡単な方法で制御することができる簡単な方法に関する。

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【0060】

薄い高分子電解質壁の透過性を熱により簡単に切り替えられることが判明した。これはおそらく高温でカチオンとアニオンとの結合がより強く分離し、次いで形成された電荷が水和し、かつポリマー鎖が絡み合うことにより壁が一次的に膨潤することによって生じる。このプロセスの可逆性は中空の高分子電解質カプセルを、薬剤のカプセル封入のため、ならびに活性物質の制御されたインテリジェント放出のための大きな高分子、ポリマーまたはナノ粒子で充填するための優れた手段を提供する。たとえば農業では多くの害虫は主として高温で活動的である。このようなカプセルに農薬をカプセル封入することにより高温かつ好天の日によりのみ放出することを保証することができる。またはインテリジェント衣類の新しい世代では、著しく発汗した場合によりのみ消臭剤が放出される。

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【0061】

有利には50℃まで、より有利には40℃までの温度で透過性は低く（閉鎖された状態）、その一方で50℃を上回る温度、特に少なくとも60℃で透過性のカプセル（開放された状態）が得られる。温度を上げることによって簡単な方法で透過性の増大を達成することができる。

【0062】

本発明のもう1つの実施態様では、高分子電解質多層カプセルの透過性を、溶剤組成または溶剤濃度の変更により修正する。従って放出挙動は溶液により調節することができ、その際、溶液としてたとえば極性の溶液、特に水および／または極性の溶液もしくはその混合物を使用することができる。放出挙動を調節するための適切な溶液はアルコール／水混合物であり、その際、アルコール成分、たとえばメタノール、エタノールまたはプロパノールを個々の適用に適合させることができる。

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【0063】

さらに高分子電解質多層カプセルの透過性は界面活性剤、たとえばドデシル硫酸ナトリウム（SDS）またはジパルミトイル-DL- α -ホスファチジルコリン（DPPC）を周囲の媒体に添加するか、またはカプセルの製造において使用されるコアのための被覆として使用することによりさらに影響を与えることができる。

【0064】

高分子電解質多層カプセルの透過性は光によっても影響を与えることができる。電解質層中の感光性の基、たとえばアゾ化合物または有機ビスアジドは、光によって化学的に変性することができ、これは光に曝露することによって架橋する感光性組成物を選択する場合、たとえば透過性の増大または透過性の低減につながる。

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【0065】

上で説明したように、本発明によればさらなる成分を高分子電解質層に挿入して透過性を広い範囲で変更することが可能である。さらなる成分はたとえば高分子電解質に共有結合していてもよいし、または高分子電解質層中に埋め込まれているのみの独立した分子もしくは粒子であってもよい。さらなる成分は高分子電解質と一緒に、または独立したユニットとして種々の方法、有利にはLbL法、あるいはまた一段法またはその後の拡散を使用して配合することができる。

【0066】

本発明によりその透過性を制御することができるマイクロカプセル、特に中空のマイクロカプセルは、有利には反対電荷の高分子電解質を可溶性のコロイドテンプレート上に交互に堆積させることによって製造することができる。高分子電解質カプセルはたとえばテンプレート、たとえば60nmから、有利には100nmから10μmまで、有利には2μmまでの大きさのテンプレートをポリカチオンおよびポリアニオンの交互の層によって被覆することによって製造することができる〔6、7〕。十分な厚さの高分子電解質壁を形成した後で、テンプレート粒子を溶解することができ、かつ中空のカプセルが得られる。テンプレートはたとえばpHを変えることにより、またはテンプレートを酸化分解することにより除去することができる。これはカプセル壁が分子量が10000より小さい、有利には5000より小さい、および特に1000より小さい小分子に対して透過性であるが、しかしポリマーに対しては透過性でないという顕著な特性によって可能である。テンプレートはカプセルの大きさと形とを決定する。500nm～10μmの範囲の単分散性カプセルを製造することができる。適切なカプセルおよびその製造および組成はたとえばWO99/47252、WO99/47253、WO00/03797およびWO00/77281に記載されており、これらの開示内容をここで参照することによって取り入れる。

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【0067】

カプセル壁の顕著な特徴は、層の材料および／または数を変えることにより、および／または環境条件、たとえばpH、塩濃度、イオン強度、イオン組成、溶剤濃度、溶剤組成および／または温度を変えることによりその特性を広い範囲で調整できることである。これ

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らのカプセルは薬剤、酵素、DNA、無機塩類、染料、ポリマー、タンパク質および／または活性な高分子のナノスケールのカプセル封入における広い展望を提供する。

【0068】

カプセルは有利には反対電荷の高分子電解質をコロイド粒子の表面上に交互に吸着させることにより製造する。0.06、有利には0.1から10 μm までで変化する大きさを有する異なったコア、たとえば無機コロイド粒子、生物細胞、タンパク質集合体、薬剤ナノ結晶を使用することができる。中空カプセルは引き続きコアを溶解することにより製造することができる。

【0069】

カプセルの透過性は、たとえばバイオテクノロジー、医薬、食料品産業などに関連する種々の範囲におけるその著しい重要性のために本質的に興味深い。 10

【0070】

本発明を以下の実施例および図面により詳細に記載する：

図1：高分子電解質多層堆積プロセスおよびその後のコアの溶解の図。最初の工程（A～D）はフルオレseinコア上での段階的なシェルの形成に関する。所望の数の高分子電解質層が堆積した後で、被覆された粒子をpH8にさらし（E）、かつバルクへのフルオレseinの浸透によるコアの溶解が開始され、その結果、最終的にコアは完全に溶解し、かつ中空のカプセルが残留する（F）。

【0071】

図2：経過時間によるフルオレseinの増加。これは異なった厚さのシェルにより被覆されたフルオレsein粒子の溶解により得られる（9、13、15および18層）。 20

【0072】

図3：17のPSS／PAH層により被覆されたフルオレseinコアの溶解の3つの段階。

【0073】

図4：層の数の相関としてのフルオレseinの拡散。

【0074】

図5：高分子電解質多層カプセルへのFITC-デキストラン（分子量75000）の透過およびカプセル封入。左側のpH=10、中央のpH=3、右側のpHはpH=3でカプセルにFITC-デキストランを充填した後でpH10に増大させた。バルクFITC-デキストランをpH=10で洗浄することによって除去した。上段は図、中央はカプセルの共焦点画像、下段は共焦点画像において示したラインに沿ったフルオレseinプロファイルを示す。 30

【0075】

図6：共焦点レーザー走査型電子顕微鏡画像：これは次のものを示す：a) 0.05 MのNaClの存在下に洗浄およびコアの溶解を実施したカプセル；b) それぞれの堆積工程の後に純水で洗浄したカプセル； 10^{-3} MのPAH-RhO溶液中で24時間インキュベーション；c) b)においてと同じ手順であるが、 10^{-2} Mの塩の存在下でのインキュベーション。 40

【0076】

図7：塩濃度に依存する、第10層のPAH-fluoおよび第12層のPAH-rhoを有するカプセル中でのフェルスター共鳴エネルギー移動効率。該図面はこれらのカプセルの典型的な蛍光スペクトルを示す。励起は495 nmに設定した。

【0077】

図8：塩濃度の関数としてのFRET効率の時間に依存する低下。

【0078】

図9 a)：図9 b)に記載のプロトコルに従って 5×10^{-3} MのPAH-rhoで充填したカプセル。カプセル封入されたPAH-rhoの放出後に同じカプセルを 3×10^{-2} Mの塩溶液によって誘発。

【0079】

図10：塩濃度の変更により透過性を切り替えることによってマイクロ分子をカプセル封入および放出する図。

【0080】

図11a)： 10^{-3} Mのフルオレセイン-PAH（分子量70000 g/mol、ポリマーの排除）の存在下でのカプセルのCLSM画像；b) 10^{-3} Mのローダミン-デキストラン（分子量50000 g/mol、蓄積）の存在下でのカプセルのCLSM画像。

【0081】

図12a)：23℃で 10^{-3} Mのローダミン-PAH（分子量70000 g/mol）の存在下でのカプセルのCLSM画像；b) 溶液を60℃で20分加熱し、かつ引き続き水で洗浄した後にカプセル中に封入されたローダミン-PAH。

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【0082】

図13：標識されたデキストラン、分子量77000に対する、低いpHおよび高いpHでの8層のPSS-PAHからなるカプセルの透過性。

【0083】

図14：異なった数のPSS-PAH高分子電解質層により被覆されたフルオレセインコアの溶解の特性時間。

【0084】

図15：高分子量の分子の透過。

【0085】

第1表：PAH-rho、 5×10^{-3} Mの溶液で充填したカプセルの百分率。24時間のインキュベーション時間後および異なった塩濃度の存在下。

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【0086】

実施例

例1：高分子電解質層の数の変更

材料

高分子電解質。ポリ（スチレンスルホン酸）ナトリウム（Na-PSS、分子量～70000）、ポリ（アリルアミン塩酸塩）（PAH、分子量～50000）およびフルオレセイン、ナトリウム塩をAldrichから入手した。エタノール、塩化ナトリウム、硼酸および塩酸をSigmaから購入した。全ての材料をそれ以上精製することなく使用した。

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【0087】

全ての実験で使用した水は3段階のMillipore Milli-Q Plus 185精製システムで準備し、かつ18.2 MΩよりも高い抵抗率を有していた。

【0088】

方法

エタノール1部およびpH2の塩酸4部を、フルオレセイン15 mg/mlの水溶液1部に添加することによりフルオレセイン粒子を準備した。光学顕微鏡により粒径を測定した。450 gで遠心分離サイクルを5回繰り返すことによって直径4～9 μmのフルオレセイン粒子を塩酸中で洗浄した。予めコアが分解することを予防するために全てのその後の多層の堆積および洗浄を塩酸中、pH2で実施した。

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【0089】

高分子電解質多層アセンブリ。この多層アセンブリは0.5 MのNaCl中、 10^{-2} Mのモノマー濃度、pH2で高分子電解質の吸着により行った。反対電荷を有するタイプの高分子電解質を引き続き、フルオレセイン粒子の懸濁液に添加し、次いで塩酸中で遠心分離サイクルを繰り返した〔7〕。フルオレセイン粒子を15分間、高分子電解質溶液と相互作用させた。フルオレセイン粒子を700 gで10分間遠心分離した。穏やかに振とうし、次いで超音波を1分間使用して遠心分離後に粒子を分散させた。

【0090】

蛍光分光分析。コアの溶解は H_3BO_3 -NaCl-NaOH緩衝液中、pH8で行った。動態は522 nmでの発光の時間依存性を記録することによって追跡した。励起は48

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8 nmに設定した。

【0091】

共焦点顕微鏡分析。ライカ（L e i c a）の共焦点走査型システムによってコアを溶解した後のカプセルの共焦点画像が得られた。開口数1.4を有する100倍の油浸対物レンズを使用した。

【0092】

図1はフルオレセイン粒子のカプセル封入および放出の図を示す。L b L吸着の後（図1 A～1 D）、pHをpH2からpH8に変更することによってコアの溶解を開始し（図1 E）、かつ一定の期間の後に完了した（図1 F）。

【0093】

フルオレセイン粒子はpH8で迅速に溶解した。考えは、こうして粒子を高分子電解質多層によって被覆することによりコアの溶解速度を遅くするというものであった。異なった数の層からなるシェル壁を製造し、かつそのフルオレセイン透過挙動に関して試験した。蛍光分光分析はコアの溶解速度を決定するために便利な手段である。というのは、コアの蛍光は染料の自己消滅の帰結として完全に抑制されるからである。染料をバルク中へ放出するにあたり蛍光強度が増大する。こうしてコアの溶解速度は試料中の蛍光の増加を測定することによって直接追跡することができる。

【0094】

図2にはpHを8に切り替えることによって得られる典型的な時間依存性の蛍光曲線が示されている。異なった厚さの層（9、13、15および18層）により被覆されたフルオレセイン粒子を、裸のフルオレセイン粒子の溶解を示す対照と比較する。

【0095】

図3に示されているように、比較的短い誘発期間（1）の後で、溶解の速度は一定になり（2）、その後、最終的にバルク中の蛍光は横這いになる（3）。初期のより緩慢な蛍光の増加はコアの溶解の開始と関連する。プロセスのこの段階で高分子電解質多層の構造は、溶解したフルオレセイン分子に由来する発生期の浸透圧により変化することがある。コアの溶解が開始したすぐ後にカプセル中のフルオレセインの濃度は一定で、かつほぼ飽和状態となる。というのも、コアの溶解および透過の進行の間での安定した状態の状況が確立されるからである。さらに、シェルの内部およびバルクとの間での一定の濃度勾配が想定される。というのは、バルク溶液が無限に希釈されることが想定されうるからである。従ってフルオレセインが高分子電解質層を通過してバルクへと浸透する速度は一定となる。実際、蛍光の直線的な増加が観察される（2）。この状態は図1 Eに示した溶解の状態に相応する。直線的な領域の勾配は高分子電解質層の数と共に低減する。明らかに吸着された層の数の増加はフルオレセインの浸透を低減する。コアが完全に溶解した後で、シェル内のフルオレセイン濃度はバルクと平衡する。拡散のための駆動力は低減し、かつ放出は横這いになる（3）。

【0096】

図4は層の数の関数としての透過率×厚さの挙動を示している。

【0097】

例2：pHの相関としての透過率

全てフルオレセインイソチオシアネートにより標識されたポリ（スチレンスルホン酸）ナトリウム（Na- PSS、分子量～70000）、ポリ（アリルアミン塩酸塩）（PAH、分子量～50000）、デキストラン（分子量～75000および4000000）およびウシ血清アルブミンをA l d r i c hから入手した。中空の高分子電解質カプセルをpH=7で濾過プロトコルを使用して8層のPSS/PAHを直径5.2 μm（micro particles GmbH社、ベルリン）のMF粒子状に交互に吸着させることにより製造した（15）。コアをpH=1で溶解した。引き続き50 mMのNaCl中で洗浄した。共焦点レーザー走査型顕微鏡（TC S L e i c a）を使用して共焦点画像を撮影した。励起波長は488 nmであった。カプセルをFITC標識した、濃度1 mg/mlのポリマー溶液中に懸濁させた。

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【0098】

F I T C 標識したデキストラン（分子量約 7 5 0 0 0 を有する）のための 5 . 2 μ m のメラミンホルムアルデヒド（M F）粒子をテンプレートとして使用し、8 層の P S S / P A H からなる中空高分子電解質カプセルの排出特性を、p H の相関として調査した。図 5（左）は、p H = 1 0 での F I T C - デキストランの存在下でのカプセルの共焦点画像を示している。カプセルの内部は暗いままであるが、背景は蛍光発光している。このことは、この条件でカプセル壁が F I T C - デキストランに対して透過性でないことを示している。変形したカプセルさえもその内側に蛍光を示さない。しかし、p H = 3 でカプセルの内部はバルクと同様に蛍光発光する（図 5、中央）。これはこの低い p H 値で F I T C - デキストランのためにカプセルが開放されることによってしか説明できない。

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【0099】

比較的低い p H および高い p H でそれぞれ「開放」および「閉鎖」された状態はカプセルの 9 0 % 以上に関して観察されたことを言及しておく必要がある。F I T C - デキストランに対して開放された状態は 6 までの p H 値で観察された。p H 8 からカプセルのほとんどは閉鎖されている。その間の p H 値、つまり p H = 7 では、開放されたカプセルと閉鎖されたカプセルとが同時に存在していた。

【0100】

充填の可能性は図 5（右）に記載されており、その際、該カプセルはまず p H = 3 で F I T C - デキストランに曝露される。次いで p H 値を 1 0 へシフトさせ、かつ F I T C - デキストランの残りを遠心分離によりバルクから除去する。カプセルが共焦点画像による蛍光プロファイルによって示されているように蛍光材料で充填されたまま残ることは注目すべきである。カプセルの内部は明るく、かつ時間の経過において一定した蛍光が認められる一方で、溶液からの蛍光シグナルは存在しない。

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【0101】

p H の変更によりカプセル壁の透過率を変え、ひいてはカプセルの充填を変える類似の実験を分子量 2 0 0 0 0 0 0 の F I T C - デキストランおよび F I T C 標識されたウシ血清アルブミンを使用して実施した。その結果は分子量 7 5 0 0 0 の F I T C - デキストランのものと同様であったが、ただし閉鎖状態と開放状態との間の移行の p H 値が 1 ~ 1 . 5 p H 単位で異なっていた。

【0102】

例 3：塩濃度によるカプセル壁透過率の制御

2 つの異なった種類の高分子電解質カプセルをこの透過率の実験のために使用した。ポリスチレンスルホン酸ナトリウム P S S およびポリアリルアミン P A H の 8 つの層を、弱く重合したメラミン-ホルムアルデヒド樹脂からなる直径 4 . 7 μ m の単分散性テンプレート上に吸着させた。1 つのバッチではコアの溶解を p H 1 で実施し、かつその後の洗浄を 0 . 0 5 M の N a C l の存在下で実施した。このプロトコルによりしわのあるカプセルが得られた（図 6 a）。これらのカプセルを 2 つの付加的な層で被覆し、かつ引き続き M i l l i p o r e 水（ $\delta < 1 8$ M Ω c m）で洗浄した。この処理の後、カプセルは球形であることが想定される。

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【0103】

第二のバッチ中のコアを 0 . 1 M の H C l 中に溶解し、かつその後、塩の添加を回避するため M i l l i p o r e 水で洗浄した。後者のプロトコルにより球状の形を有するカプセルの均一な集団が得られた（図 6 b）。2 つのタイプのカプセルの透過挙動は類似していた。2 つ目の種類のカプセルにより得られた結果を本質的にこの例で報告する。付加的な 2 つの層を有するカプセルにより得られた顕著に異なった結果が得られる場合にのみ言及する。

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【0104】

カプセル壁の透過性を蛍光標識したポリマーのポリスチレンスルホネート P S S（1 2 0 0 0 0 g / モル）、ポリアリルアミン P A H（7 0 0 0 0 g / モル）、デキストラン（5 5 0 0 0 g / モル）およびヒトのアルブミン（7 0 0 0 0 g / モル）を使用して調査した

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。245番目の位置ごとにローダミンBで標識したポリアリルアミンPAHを使用した。フェルスター共鳴エネルギー移動の測定において、120番目の位置ごとにフルオレセインで標識されたポリアリルアミンPAHを使用した。カプセルをそれぞれのポリマー試料の 5×10^{-3} M溶液に添加し、その濃度を常にモノマー単位で表現する。引き続き共焦点画像によりポリマーの透過を追跡した(TCSCN Leica、ドイツ)。カプセルの内側および外側の蛍光分子を定量化した。

【0105】

図6bにおける画像は、カプセルの赤道面の共焦点スキャンを示しており、該カプセルは標識されたポリマー溶液と共に24時間インキュベーションしたものである。カプセルの約90%がポリマーを排除した。ポリマーで充填したカプセルの小数が破損し、かつ大きな穴が観察され、この穴を通過してポリマーはほぼ即座にカプセル内部へと拡散することができたであろう。

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【0106】

次いで0.1 MのNaClの存在下で類似の実験を実施した。共焦点画像(図6c)は、全てのカプセルの内部がバルク中に存在するのとはほぼ同じ濃度のポリマーを含有していることを示している。この発見は塩の存在下でのポリマーに対する高分子電解質壁の透過性の増大と一致する。この「壁の開放」は、プラスおよびマイナスに荷電したポリマーに関しても、ならびに中性のポリマーであるデキストランに関しても観察された。従って高分子電解質試料の構造における、塩により誘発される変化、たとえば変化した種類のらせん化(coiling)は浸透の原因となるメカニズムではあり得ない。むしろ壁中の高分子電解質錯体の構造および特性の、塩により誘発される変化を、透過率における著しい増加の原因であると見なすべきである。この画像から明らかであるように、蛍光試料分子はカプセル壁の内部の表面上または内側に蓄積した。このことは、プラスに荷電したカプセル表面上に吸着していたPSSに関して予測されたが、しかし標識されたPAHに関してカプセル壁中のPAH分子の交換か、または内側のPSS層への吸着が推測されなくてはならない。

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【0107】

次いで塩濃度への依存性において透過率を調査した。カプセルを 5×10^{-3} MのPAH-ローダミンと混合し、かつ塩化ナトリウムの濃度を上昇させた。分散液を10分間インキュベーションし、かつ内部のポリマーの量をバルク中のポリマー濃度と比較した。 5×10^{-3} MのNaClまではカプセルへのポリマーの浸透は検出されなかったが、しかしより高い濃度に関して、カプセルは次第にPAH-Rhに対して透過性になった。 2×10^{-2} MのNaClで、PAH-Rhの浸透の時間を経時的な共焦点画像撮影により推定した。塩の添加と、カプセルの内側および外側のポリマー濃度の完全な平衡との間の時間は、8層からなるカプセルに関して約6分であった。20分以内に生じるより遅い浸透を、10層のカプセルに関して測定した。共焦点画像はそれ以上高い濃度における動態を追跡することができなかった。というのは、拡散およびポリマー濃度の平衡が早すぎたからである。

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【0108】

ポリマーの浸透を誘発するための塩の最小量は、カプセルをポリマー溶液中、種々のNaCl濃度で24時間インキュベーションすることにより測定した。次いで充填したカプセルの百分率を、共焦点画像中で、そのつど約100個のカプセルに関して充填されたカプセルと空のカプセルの数とを数えることによって決定した(第1表)。 10^{-2} Mの塩溶液において、全てのカプセルが標識されたポリマーを含有していた一方で、 0.5×10^{-2} Mの塩溶液中ではカプセルのわずか16%が24時間以内に充填されたにすぎなかった。さらに低いNaClで、カプセルはポリマーに対して不透過性のままであった。従って高分子に対するカプセルの透過率の増大は、 $0.5 \sim 2 \times 10^{-2}$ Mの比較的狭い範囲の塩濃度内で生じた。

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【0109】

さらに、塩を添加した後の透過の速度を制限するプロセスが、カプセル壁中の塩により誘

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発される構造の変化が壁をより透過性に行っていることにより生じるのか、または層を通過してポリマーが拡散することにより生じるのかを試験した。このために、PAH ($M_n = 70000$) の透過を、 10^{-2} M の塩 (NaCl) および 5×10^{-3} PAH を同時に添加した直後に、またはサンプルを 10^{-2} M の NaCl 中で 12 時間インキュベーションし、かつその後はじめて PAH を添加した後に観察した。第二の事例では、特徴的な透過時間は約 30 分であることが判明し、これは NaCl およびポリマーを同時に添加した場合に観察される約 50 分の特徴的な透過時間よりも短い。これらの数は 2 倍、顕著である。これらは、1) 観察された条件下でのポリマーの透過は、濃度平衡に関して少なくとも 30 分を必要として全体的に遅い、および 2) 塩自体による透過性の誘発は数分を必要とする、ということを示している。

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【0110】

カプセル壁を通過するポリマーの透過のメカニズムに関して、2つの極端な状況を区別することができる。塩溶液中でのインキュベーションが壁を通過した水によって充填された気孔の形成につながり、該気孔を横断してポリマーはカプセルの中および外へと拡散することができるか、または溶液中のイオンの存在により PSS および PAH の静電結合が弱くなり、一次的な結合の破壊および再形成によって網状構造を通過してポリマーの拡散が可能になる。pH または塩により誘発される気孔の形成は、弱い高分子電解質からなる顕微鏡的に平坦な膜に関して観察された。これらの比較的大きな気孔は 10^2 nm のオーダーの半径を有していた。従って SFM はカプセル分散液を H_2O 中乾燥させることにより、または 0.1 M の NaCl 中で雲母支持体上に施与することにより製造された高分子電

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【0111】

ポリマーの浸透は水で充填された気孔を通過する拡散により生じると想定すると、見かけの気孔断面積は 1000 nm^2 のオーダーである。これは半径 17 nm の 1 つの気孔または 2 nm の 80 の気孔にそれぞれ匹敵する。いずれの場合でも気孔面積はカプセル表面の極めて小さい面積割合を構成しているにすぎないことに言及するに値する。これらのことを考慮すると、これらの気孔の存在が確実であるとしても、SFM によって該気孔を検出することはほとんど不可能であることが明らかになる。

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【0112】

バルク電解質の塩濃度への依存性におけるカプセル壁の形態の変化を発見するために、蛍光標識した高分子電解質の間でフェルスター共鳴エネルギー移動 FRET を適用した。カプセルは PSS、PAH-フルオレセイン、PSS、PAH-ローダミン、PSS、PAH の順で 6 つの高分子電解質層を与えることにより被覆した。ポリマーは 0.5 M の NaCl の存在下で吸着されたが、しかし後から該カプセルを水で十分に洗浄した。異なった濃度の NaCl 溶液を添加した 5 分後にカプセル分散液の FRET を測定した。励起は 495 nm に設定し、その際、フルオレセインのみが光を吸収した。ローダミン分子がフルオレセインの近く (6 nm まで) に位置する場合、共鳴エネルギー移動が行われ、かつローダミンの蛍光も観察することができる (図 7、挿入図)。

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【0113】

相対移動効率 E_{FRET} を $E_{FRET} = (I_{rh} - 0.4 I_{fl}) / (0.6 I_{fl} + I_{rh})$ として定義し、その際、 I_{fl} はフルオレセイン発光における最大値に相応する 522 nm での蛍光強度であり、かつ I_{rh} はローダミン発光に相応する 582 nm での強度である。

【0114】

ファクター 0.4 はアクセプタの発光波長でのドナーの蛍光を考慮に入れる。塩濃度に対する E_{FRET} のプロットは移動効率における明確な低減を示し (図 7)、これはまさにカプセル壁が PAH に対して透過性になる濃度範囲で生じる。より高い塩濃度では、 E_{FRET} はほぼ一定に保たれる。従って、FRET は 10^{-2} モル/L 近辺の狭い塩濃度範

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囲内で生じる高分子電解質壁のトポロジーにおける顕著な変化を報告すると結論づけることができる。塩濃度による E_{FRET} の低下はローダミンおよびフルオレセインの染料分子の間の距離が増大することを示唆している。 $FRET$ 信号は、カプセルがふたたび水に曝露されると、5分以内に完全に回復する。これらの調査結果は高分子電解質層の若干の可逆的な膨潤と明らかに一致する。

【0115】

壁の開放の透過性の増大と $FRET$ の低減との間の相関関係は、透過するポリマーの不在下での、塩により誘発されるカプセル壁の変化の特徴付けを考慮に入れる。後者は壁の特性に若干の影響を与えた、特により顕著な量の透過ポリマーが壁に吸着されたことが考えられる。カプセルを $1.5 \times 10^{-2} M$ の $NaCl$ 中に導入し、かつ E_{FRET} を時間への依存性において測定した (図8)。20分後、 $FRET$ を $E_{FRET} = 0.31$ の値で飽和させた。変化の時間の尺度は共焦点顕微鏡分析により観察される透過率の変化と一致していた。 $FRET$ 信号の変化の時間および程度はいずれも塩の濃度により増大した壁の構造の変化に関連する。

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【0116】

狭い塩濃度範囲で生じる、両方の層の透過率を増大し、かつ蛍光エネルギー移動を低減するメカニズムおよび駆動力を以下に論じる。比較的遅い透過は、水により充填された気孔が透過のための基本的な経路であることが想定される場合、極めて小さい気孔面積を示唆している。その一方で、 $FRET$ 信号の電荷は標識されたポリマーの大部分に影響を与える構造的な変化を想定するものであると理解することができるのみである。従って塩は層の変化全体を示している。これは小数の気孔の局在した形成を想定することとほぼ一致しない。従って我々は透過率の増大は溶解度の増大または塩濃度の上昇により生じる透過性の種類と層ポリマーとの相互作用の結果であると理解する。

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【0117】

より高い塩濃度が層の構造を柔軟にすることが判明した。というのも、バルク中で利用される遊離イオンが層中の電荷を遮閉するために、ポリアニオンおよびポリカチオンの間に見られるイオン対が部分的に開放されるからである。従って層の膨潤はポリマー対の分子の弾性の緩和であると理解することができ、これは層ポリマーのよりいっそうかさ高な配置につながる。 $10^{-2} M$ の $NaCl$ でデバイ遮閉距離は、ポリアニオン/ポリカチオン対の層厚さの増分と十分に比較してちょうど $3 nm$ であることは言及に値する。より低い電解質濃度で、静電相互作用は、層全体にわたる静電相互作用を生じるより大きな範囲を有する。

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【0118】

数多くの適用にとって重要な問題は、透過性の切り替えプロセスの可逆性である。高い塩濃度でカプセルを製造し、かつそれにも関わらず洗浄後に高分子に対して不透過性であるという事実は、カプセルの開放および閉鎖の可逆性を示している。さらなる実験においてカプセル壁を閉鎖する時間を決定する。 $3 \times 10^{-2} M$ の $NaCl$ 溶液をカプセルに30分間適用した。その後、純水中で約 $5 \times 10^{-3} M$ の $NaCl$ に希釈した。10分後、カプセルは共焦点画像により観察されるように PAH -ローダミンに対して不透過性になった。 $FRET$ 信号は5分以内に回復した (図8)。

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【0119】

高分子電解質に関する壁透過性の変更の可逆性は、カプセル封入の際に適用されるアグレッシブな物質、溶剤、 pH または熱により生じる化学的な負荷を回避しながら、高分子、たとえばポリペプチド、 DNA 、酵素またはポリマーの容易かつ柔軟なカプセル封入を可能にする。しかし高い塩濃度で高分子によりカプセルを充填し、引き続き水で洗浄することにより、内部にカプセル封入されたポリマーは少量得られるにすぎない。おそらく、高分子は閉鎖された壁よりも早く洗い流されるのであろう。

【0120】

しかしより効率の高い充填は、カプセル封入のためのポリマーが、塩の除去により誘発される壁の閉鎖の間にバルク中に存在する場合に達成される (図10)。この方法によりカ

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プセルは 5×10^{-3} M の PAH-Rh 溶液により充填される (図 9)。PAH-Rh 溶液の共焦点画像の蛍光強度を標準とすると、カプセル封入されたポリマーの量を決定することができた。内部の PAH-Rh の濃度の平均は初期の溶液の約 50 % であった。

【0121】

負荷されたカプセルのもう 1 つの重要な特性は、その放出挙動である。負荷されたカプセルを水 2 ml 中または 3×10^{-2} M の塩溶液 2 ml 中で 30 分間インキュベーションした。カプセルを遠心分離により試料から除去し、かつ両方の溶液中の PAH-Rh の濃度を蛍光分光分析により測定した。

【0122】

測定を実施する前に塩不含の溶液を後から 3×10^{-2} M に調整することにより塩濃度へのローダミン発光の依存性を考慮した。 10

【0123】

純水中に放出される PAH の量は検出することができなかったが、その一方でほぼ全てのカプセル封入された PAH-Rh は 3×10^{-2} M で放出された。共焦点画像は、これらのカプセルの内部がふたたび蛍光ポリマー不含であることを証明した。

【0124】

例 4：温度依存性の透過性

弱く重合したメラミン-ホルムアルデヒド樹脂の単分散性の球体 ($d = 5.94 \mu\text{m}$) をテンプレートとして使用した (Microparticle GmbH 社)。粒子は 0.1 M の塩化ナトリウム中の 10^{-2} M の PSS の水溶液から、ポリスチレンスルホン酸ナトリウム PSS (分子量 70000 g/mol 、Aldrich 社) およびポリアリルアミン塩酸塩 PAH (分子量 70000 g/mol 、Aldrich 社) を交互に吸着させることにより被覆した。8 層後に 0.1 M の塩酸中でコアを溶解した。酸および 10^{-2} M の NaCl の水溶液により洗浄することによって分解生成物を除去した。中空の高分子電解質カプセルが残った。溶解の間の機械的負荷により生じたカプセル壁における若干の欠陥を除去するために 2 つの付加的な層をコアの溶解後にカプセル上に被覆した。その後、Milli-Q-water (抵抗率 $> 18 \text{ M}\Omega \text{ cm}^{-1}$) を用いてカプセルを十分に洗浄した (8 回)。この手順により塩およびプロトンからの痕跡を本質的に除去し、かつ pH 値は 6 になった。 20

【0125】

透過率の調査のために蛍光標識したポリマーを使用した。PAH (分子量 70000、245 番目の PAH ユニットあたり 1 つのローダミン分子、および分子量 15000、320 番目の PAH ユニットあたり 1 つのローダミン) および PEI (分子量 2000、650 番目の PEI ユニットあたり 1 つのフルオレセイン分子) をローダミンイソチオシアネートまたはフルオレセインイソチオシアネートとの反応により標識した。スチレンスルホン酸とメタクリロイルオキシエチルチオカルバモイルローダミン B (Polyscience) の共重合により、標識した PSS (600 番目のポリマーユニットあたり 1 つのローダミン分子) を合成した。ポリマーの分子量はゲル透過クロマトグラフィーによれば 120000 g/mol であることが測定された。フルオレセイン標識したヒト血清アルブミン (分子量 69000) およびローダミン標識したデキストラン (分子量 40000 g/mol) を Aldrich 社から購入した。 40

【0126】

レーザー走査型顕微鏡分析をカプセルの充填度の測定のために実施した。40 倍または 100 倍の油浸対物レンズを有する逆リサーチ顕微鏡 (inverse research microscope) (Leica、ドイツ) を使用した。内部のポリマー/モノマーの量を、カプセル内部の蛍光強度の積分により測定した。正確な濃度を補正曲線から把握し、それぞれの蛍光試料に関して別々に決定した。カプセルに関する透過性の広い分布に基づいて、そのつど 5 つのカプセルから統計学的値をとった。明らかに破壊されていると認識できたカプセル (約 20 %) は、測定から除外した。テンプレート依存性の測定は試料の上の手製のアルミニウム装置で行い、これにより温度は電子制御装置により設定した 30

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【0127】

走査型顕微鏡分析をカプセルに対して適用し、雲母支持体上、空気で乾燥させた。60℃での熱処理後、カプセル溶液の液滴を雲母表面に移し、かつ温度の変化なしで乾燥させることによって乾燥工程を行った。

【0128】

4.1 透過率

カプセルの透過率をプラス（PAH）およびマイナス（PSS）に帯電した、両性イオン性（アルブミン）および電荷を有していない（デキストラン）蛍光ポリマー（デキストラン）に関して調査した。一般にカプセルを、ポリマーユニットに関して約 2×10^{-3} モル/Lの濃度でポリマー試料と混合し、かつ15分間インキュベーションした。次いでカプセルの中心における蛍光強度を共焦点画像により測定した。カプセルの断面内での蛍光事象を積分し、かつバルク溶液中の蛍光事象と比較した。カプセルの外側よりも内側でより低い蛍光強度は、ポリマー試料に対するカプセル壁の不透過性を示している。実験の開始時点でのカプセルの内側および外側の同じ蛍光強度は、カプセル壁を通過するポリマーの迅速な浸透により生じ、これは多くの場合、破壊されたカプセルに関して観察される。以下に記載されるようなデキストランの場合、カプセルの内側の蛍光が内部におけるポリマーの蓄積を示唆するバルク相中におけるよりも高い。

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【0129】

第一にポリマー試料のためのカプセル壁の透過率を、熱処理前に調査した。カプセルの大部分は24時間以内に荷電の徴候とは無関係に、60000 g/モルを上回る分子量の帯電した高分子電解質に対して不透過性であった。図11aは70000 g/モルのPAHに関する共焦点画像を示している。帯電していないデキストランの場合、約20%のカプセルの内側のポリマーの異常な蓄積が観察され（図11b）、その一方でその他のカプセルはポリマーもまた排除する。

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【0130】

浸透に対するポリマー鎖の長さのさらなる影響を測定した。1時間以内にカプセルの80%が70000 g/モルのPAHに対して不透過性であり、約65%が15000 g/モルのPAHに対して不透過性であり、かつPEI2000に関して全てのカプセルが透過性である。小さい染料分子、たとえばフルオレセイン、ローダミンおよびアクリジンオレンジは、その電荷とは無関係にカプセル壁を通過することができる。

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【0131】

温度へのカプセル壁の透過性の依存性は、カプセルを室温から80℃まで10℃の段階ごとに共焦点画像によりオンラインで（現場で）追跡した。それぞれの段階で温度を5分間、安定させた。カプセルおよびバルク溶液中でのポリマーの分布における変化はそれぞれのポリマーに関して類似しており、かつカプセルをローダミン標識したPSSの存在下で加熱するために示した。50℃までポリマーはカプセル壁から排除されたが、しかしこれより高い温度ではPSSがカプセル中へ流入し始め、カプセルの内側で平衡またはわずかな過剰さえも達成された。浸透の速度は温度と共にさらに増大した。これは、温度を上げることにより極めて容易に大きなポリマー分子に対してカプセル壁を開放することができることの明らかな証明である。

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【0132】

さらに開放が不可逆的なプロセスであるか、または温度を50℃より低く下げることによりカプセル壁をふたたび閉鎖することができるかを試験した。これは、加熱および冷却の時間において逸脱する、異なった温度条件下でポリマーの不在下にカプセルを処理することにより調査した。

【0133】

カプセル壁透過性の増大につながる可能なメカニズムを以下に論じる。膜を通過する拡散は一般に温度と共にポリマー種の高い移動度に基づいて増大するという事実以外に、狭い温度範囲で観察される比較的強い効果は、高分子電解質壁における構造の変化を示す。1

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つの可能なメカニズムは、pHおよび塩による類似のシステム処理に関して上で記載したような、高分子電解質膜における気孔の開放であるかもしれない。PSS/PAHカプセルはより高い温度で直径において10%以上収縮することが観察された。このようなプロセスの機械的負荷は気孔の形成も生じる可能性がある。このような気孔を発見するために、カプセルを雲母支持体上で乾燥させた後、解像度の高い走査型顕微鏡によってカプセル壁を調査した。3つの試料を60℃に加熱する前、加熱中および加熱後に準備したが、しかしカプセルの直径が低減する以外の顕著な相違は観察されなかった。この実験結果にも関わらず、その後の乾燥プロセスがカプセル構造を著しく変えうるために、気孔の形成は完全に排除することができない。

【0134】

もう1つのメカニズムはポリアニオンとポリカチオンとの間の静電結合が弱くなることである。このことにより膜中の電荷密度が増大し、これはより強力な水和、膨潤および壁を通過するポリマーのより良好な拡散に関わる。14層からなり、そのうち第10層がPAH-フルオレセインであり、第12層がPAH-ローダミンであるカプセル中の共鳴エネルギー移動を調査することにより、このメカニズムを支持することが試みられた。溶液のスペクトルを60℃で20分加熱する前、加熱の間および加熱後に測定した。加熱の前および後のスペクトルはかなり似ているように見え、これは壁構造中でほぼ可逆的な変化を示していた。60℃でのスペクトルは他のスペクトルから著しく逸脱していた。ローダミン蛍光の低減は、それぞれフルオレセインおよびローダミン分子またはポリマー層との間の平均された距離の増大を示唆している。観察されたスペクトル変化の由来としての単純な加熱効果を排除するために、106番目の位置ごとにフルオレセイン分子を有し、かつ345番目の位置ごとにローダミン分子（分子量70000g/mol）を有するPAHポリマーを同じ条件下で調査した。

【0135】

調査結果はカプセル化高分子の独自の可能性を提供する。というのも、カプセル壁の透過性は温度により制御されることが判明したからである。カプセル壁は加熱により開放され、かつ周囲の材料は流入することができる。カプセルが所望の材料で充填されたら、壁は溶液を冷却することによりふたたび閉鎖することができる。その後、外側の残りの材料、たとえばポリマーを洗い流し、かつ封入された高分子はカプセルの内側に残る。カプセル封入は、不透過性のカプセル（図12a）を20分間、60℃に加熱することにより、全ての標識されたポリマーに関して首尾よく行われる。冷却および洗浄後、カプセルは、たとえば図12bに示されているようにポリマーを含有していた。本来のポリマー溶液の蛍光強度の定量的な測定および内部にカプセル封入されたポリマーは、内部に30%のバルク濃度を生じた。使用された両方のポリマー試料、つまり標識されたPSSおよびPAHが透過性の高い可逆性を示す内部に捕捉された。充填したカプセルを1月以上貯蔵する場合でも、ポリマーの損失はわずかであった。この損失は特にPAHが50%を越える場合に顕著であるが、しかしデキストランの場合はゼロであるにすぎなかった。

【0136】

【外2】

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【 0 1 3 7 】

【 表 1 】

第1表：PAD-rho の溶液、 5×10^{-3} M中および異なった塩濃度の存在下で
24時間インキュベーションした後の充てんカプセルの百分率

塩濃度 / 10^{-4} mol/L	1	5	10	50	100	200
充てんカプセルの百分率 / %	4.6	8.5	6	16.7	100	100

【図面の簡単な説明】

【図 1】

高分子電解質多層堆積プロセスおよびその後のコアの溶解を示す図。

【図 2】

経過時間によるフルオレセインの増加を示す図。

【図 3】

17 の PSS / PAH 層により被覆されたフルオレセインコアの溶解の 3 つの段階を示す図。

【図 4】

層の数とフルオレセインの拡散との相関関係を示す図。

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【図 5】

高分子電解質多層カプセルへの FITC-デキストランの透過およびカプセル封入を示す図。

【図 6】

カプセルの共焦点レーザー走査型電子顕微鏡画像を示す図。

【図 7】

カプセル中での塩濃度に依存するフェルスター共鳴エネルギー移動効率を示す図。

【図 8】

塩濃度の関数として時間に依存する FRET 効率の低下を示す図。

20

【図 9】

5×10^{-3} M の PAH-rho で充填したカプセルを示す図。

【図 10】

塩濃度によるマイクロ分子を封入および放出を示す図。

【図 11】

フルオレセイン-PAH の存在下でのカプセル (a) およびローダミン-デキストランの存在下でのカプセル (b) の CLSM 画像を示す図。

【図 12】

ローダミン-PAH の存在下でのカプセル (a) およびカプセル中に封入されたローダミン-PAH (b) の CLSM 画像を示す図。

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【図 13】

8 層の PSS-PAH からなるカプセルの透過性と pH との関係を示す図。

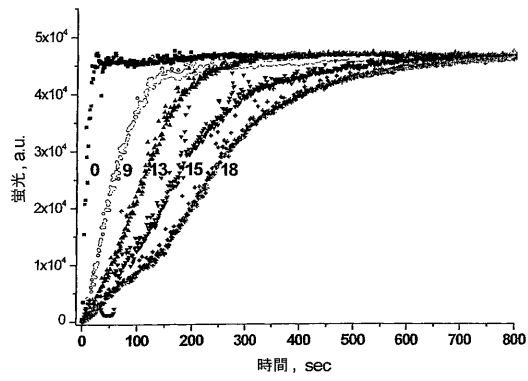
【図 14】

PSS-PAH 高分子電解質層の数とフルオレセインコアの溶解の特性時間を示す図。

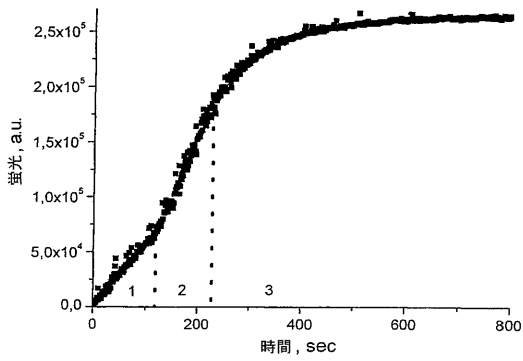
【図 15】

高分子量の分子の透過を示す図。

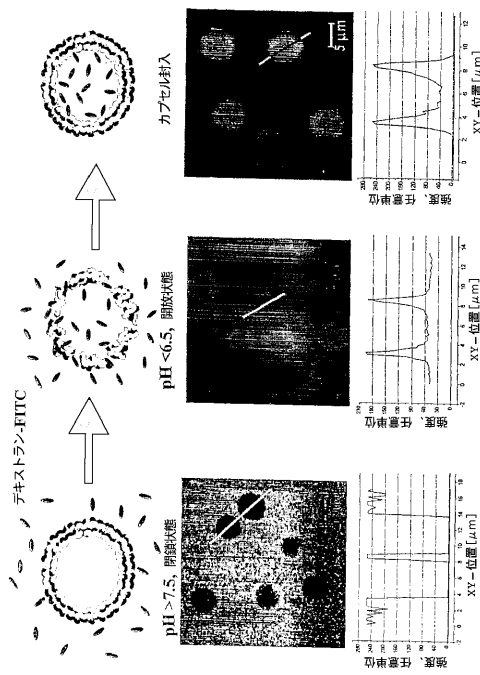
【図 2】



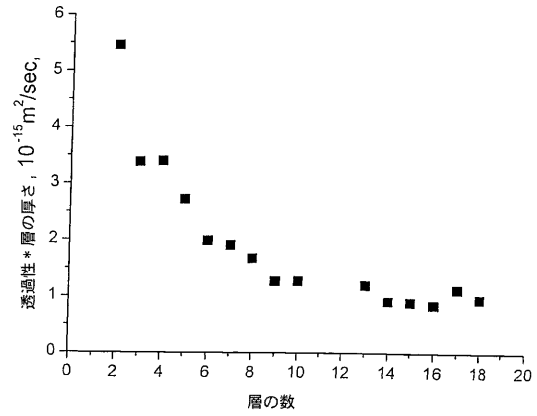
【図 3】



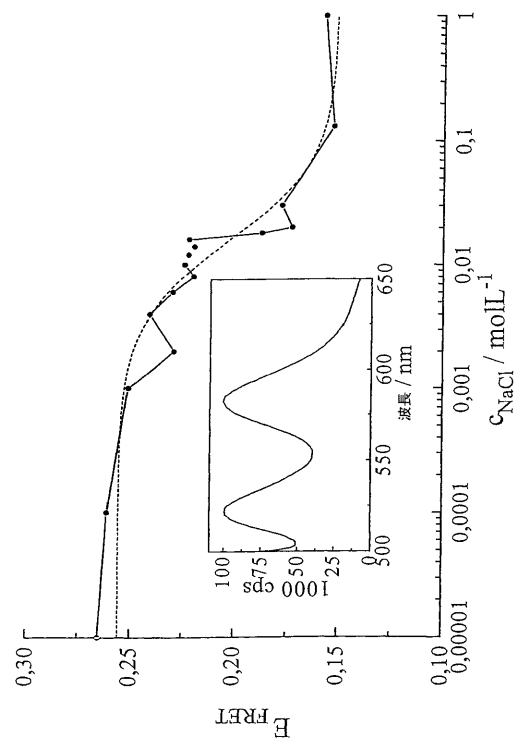
【図 5】



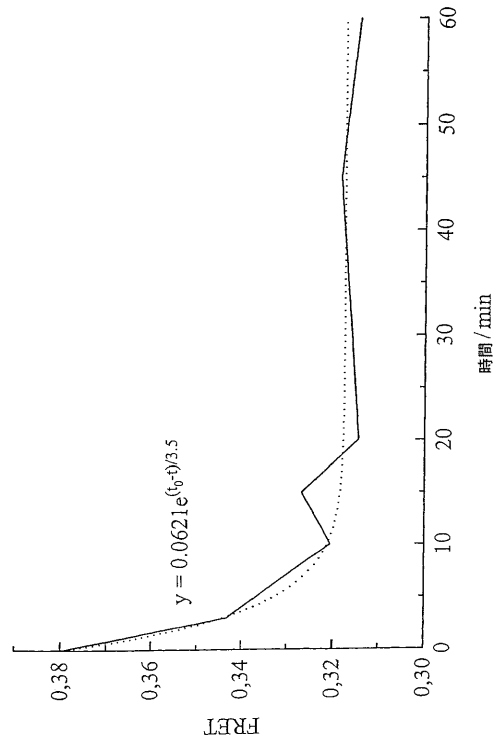
【図 4】



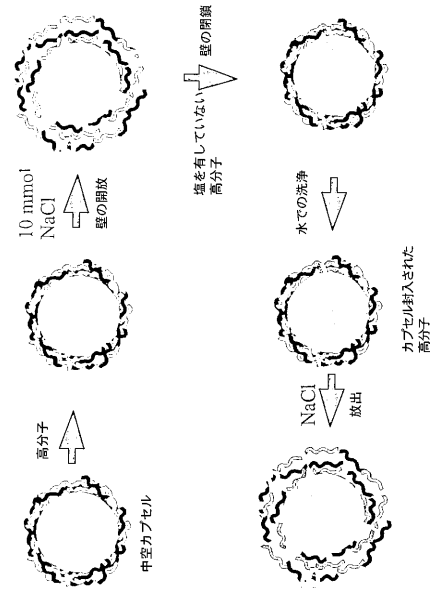
【図 7】



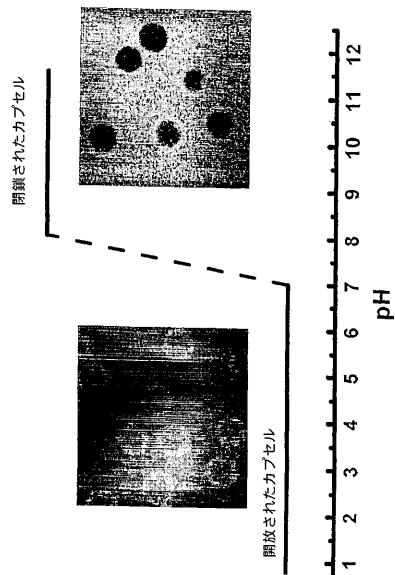
【図 8】



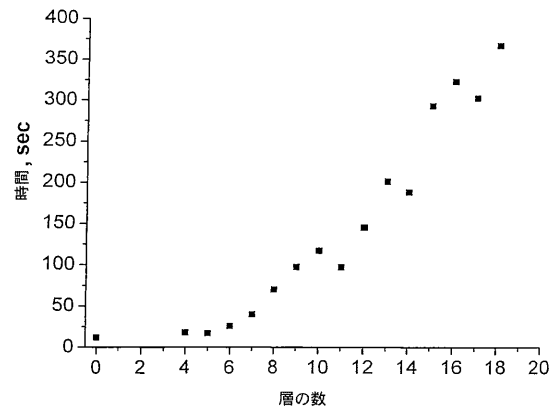
【図 10】



【図 13】



【図 14】



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- (71) Applicant (for all designated States except US): MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V. [DE/DE], Hofgartenstrasse 8, 80539 München (DE).
- (72) Inventors; and
(75) Inventors/Applicants (for US only): ANTIPOV, Alexei [RU/DE], In der Feldmark 7, 14476 Golm (DE), VIEIRA, Euridice [PT/DE], c/o Max-Planck-Institut für Kolloid- und Grenzflächen, enforshung, 14424 Potsdam (DE), IBARZ, Gemma [ES/DE], c/o Max-Planck-Institut für Kolloid- und Grenzflächen, enforshung, 14424 Potsdam
- (74) Agent: WEICKMANN & WEICKMANN, Postfach 860 820, 81635 München (DE).
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WO 02/17888 A2

(54) Title: CONTROLLED AND SUSTAINED RELEASE PROPERTIES OF POLYELECTROLYTE MULTILAYER CAPSULES

(57) Abstract: Method of layer-by-layer (LbL) assembly of oppositely charged polyelectrolytes was applied to coat fluorescein particles. These particles with a size of 4-9 µm were prepared by precipitation of fluorescein at pH 2. Polystyrenesulfonate (PSS) and polyallylamine (PAH) were used to compose the polyelectrolyte shell on the fluorescein core. The release of fluorescein molecules through the polyelectrolyte shell core dissolution was monitored at pH 8 by increasing fluorescence intensity. The number of polyelectrolyte layers sufficient to sustain fluorescence release was found to be 8-10. Sequentially adsorbed layers prolong core dissolution time for minutes. The permeability of polyelectrolyte multilayers of the thickness of 20 nm is about 10⁻⁸ m/s. Mechanism of fluorescence diffusion and osmotically supported release is under discussion. The features of release profile and possible applications of LbL method for shell formation in order to control release properties for entrapped materials are outlined. Also ambient conditions of pH, temperature and salt concentration were changed to control the permeability of polyelectrolyte multilayer capsules.

6	Description
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A major task in the development of advanced drug formulations deals with the elaboration of delivering systems providing sustained release of bioactive materials. Mostly, these delivering systems comprise polymer particles in the size range of 10^2 to 10^5 nm. The drug molecules are embedded in polymer matrices or in core-shell structures. In the latter the shell degradation rate determines the release rate of the bioactive core material.

In principle, a shell around the active core can be fabricated by adsorption of polymers or biopolymers onto the drug¹ particle surface [1] or by adsorption of the monomers with subsequent polymerization at the interface [2-4]. The composition of the shell may additionally provide certain functionalities. It may be adjusted to facilitate the interaction of the core with the solvent or to add certain desired chemical properties. The shell may also have magnetic, optical, conductive, or targeting properties for directing and manipulating the core containing bioactive material.

Recently, a novel type of shell structures constituting polymer capsules has
30 been introduced [5-7]. These novel hollow polymeric capsules have a
predetermined size in the sub-micron and micron range and tunable wall
properties [7,13-15]. These capsules are fabricated by means of layer-by-

WO 02/17888

PCT/EP01/09908

- 2 -

layer (LbL) assembling of polyelectrolytes onto colloidal particles with subsequent removal of the colloidal core. The layer-by-layer (LbL) assembling is performed by alternating adsorption of oppositely charged species, such as polyelectrolytes [16,1] onto the surface of colloidal particles. The driving force for LbL adsorption is the electrostatic attraction between the incoming polymer and the surface.

Various cores, e.g. organic or inorganic materials, biological cells, drug crystals or emulsion droplets, ranging in size from about 60 nm to tens of microns have been utilized as templates for multilayer formation by the LbL technique. Up to now a variety of different substances, such as synthetic and natural polyelectrolytes, biopolymers, proteins, nucleic acids, magnetic and fluorescent inorganic nanoparticles, lipids, etc. were employed as layer constituents to build the multilayer shell on colloidal particles. The thickness of the shell walls depends on the conditions of its preparation. It can be tuned in the nanometer range. The thickness of the multilayer films on colloidal particles can be adjusted in the nanometer range e.g. by adsorption of varying numbers of layers. It was established [8, 9] that the capsule walls have semipermeable properties. They are permeable for small molecules such as dyes and ions while they exclude compounds with a higher molecular weight [17,9].

There is a variety of materials, the encapsulation of which is desirable for application in different areas of technology, such as catalysis, cosmetics, medicine, biotechnology, nutrition and others.

One possible approach to load capsules with polymers is to embed the desired polymers within the inner layers of the shell structure while forming the capsules and to desorb polyelectrolytes, e.g. multivalent ions, from inner layers of empty shells into their interior [18]. Extraction of these ions results then in the release of the polymers into the capsule interior.

WO 02/17888

PCT/EP01/09908

- 3 -

Another approach is to directly synthesize polymers inside the capsules by taking advantage of the permselectivity of the capsule walls [19]. While capsules can be successfully loaded thereby these methods have only limited applicability with regard to the polymer species and often harsh
5 conditions have to be used for core dissolution or polymerization, being e.g. a low pH, oxidizing agents or organic solvents, which are used for core decomposition, or an elevated temperature during synthesis.

It would be desirable to provide systems having controllable or adjustable loading as well as release properties. In particular a method would be
10 favourable which allows the loading or release of materials into and from capsules by modifying the capsule wall permeability. Further, for most applications a defined and controllable permeability of the capsule wall is required in order to control the process of loading the capsules as well as
15 any subsequent release under specific environmental conditions. Due to the fact that the loading is preferably fast, but the release should be in most applications slow, it is further desired that the permeability is switchable.

It was therefore an object of the present invention to provide methods to
20 influence, vary or switch properties of capsule walls. Another object of the invention was to provide means to introduce macromolecules into the capsules and to switch and control capsule permeability for them.

These objects are solved by a process for controlling the permeability of
25 polyelectrolyte multilayer capsules by variation of the reaction conditions during the preparation or use of the capsules, characterized in that at least one of the reaction conditions of pH, temperature, salt concentration, ion composition, ion concentration, ionic strength, solvent composition or solvent concentration is varied.

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WO 02/17888

PCT/EP01/09908

- 4 -

The invention further relates to a process for controlling the permeability of polyelectrolyte multilayer capsules, wherein the number of polyelectrolyte layers is adjusted in such a way that the desired permeability is achieved.

5 According to the invention the permeability of polyelectrolyte multilayer capsules can be determined, varied or/and controlled by parameters of the environment of the capsules, e.g. reaction conditions during preparation or use of the capsules, media, in which the capsules are contained or into which the capsules are transported. It was found that the permeability to
10 high molecular weight compounds as well as low molecular weight compounds can be adjusted according to the needs in different applications.

As used herein high molecular weight compounds or macromolecules are
15 molecules having a molecular weight of at least 30 000 Da, more preferably at least 50 000 Da and most preferably at least 70 000 Da. Low molecular weight compounds or small molecules are molecules having a molecular weight of less than 10 000 Da, preferably less than 5 000 Da and more preferably less than 1 000 Da.

20 The permeability control according to the invention offers a unique tool for entrapping molecules within capsules and releasing them in a predetermined manner, e.g. over an extended period of time or at a desired, predetermined site or time point.

25 The process according to the invention enables particularly a reversible amendment of the permeability of capsules. This enables specifically charging the capsules with desired active substances or specifically releasing active substances entrapped in the capsules, respectively, by
30 amending the environmental conditions of the capsules in a simple way. Thus, e.g. a permeability increase makes it possible to later charge the finished capsules with active substances. Such an "open condition",

WO 02/17888

PCT/EP01/09908

- 5 -

wherein active substances can permeate through the capsule walls is present according to the invention when over 50%, more preferably over 70% and most preferably over 90% of the capsules are permeable. After the capsules have been charged, e.g. during storage or transport, the permeability of the capsule wall to the entrapped active substance can be reduced by adjusting of suitable conditions so that no active substance can leave the capsules. Such a "closed condition" of the capsules, however, at the same time prevents that further, possibly undesired substances can enter the capsules. At the desired time and site of release, respectively, the active agent can be released in a defined way, e.g. delayed, by increasing the permeability of the capsule walls. Further, it is possible to obtain a release in certain compartments of cells or certain areas of an organism by using capsule walls exhibiting high permeability to the entrapped active substance under conditions prevalent in the desired tissue.

According to the invention the permeability of capsule wall and thereby the incorporation or exclusion of macromolecules can be tuned by environmental conditions in a defined manner. Capsules composed of polyelectrolytes, the charge of which depends on the pH, can be used for a pH-controlled uptake and release of macromolecules.

The practical use of polyelectrolyte capsules for sustained drug release requires quantitative data on the permeation of small molecules through polyelectrolyte walls, which are presented herein.

In the light of sustained release it would be advantageous to be able to decrease the layer permeability for small polar molecules once they are encapsulated. One possible way to approach this goal is the use of lipids as a layer constituent [9, 10]. Herein, the formation of thicker capsule walls is presented in detail being a favourable and easy way to decrease permeation. It was found that increasing the layer number will decrease penetration of the shells by encapsulated material or molecules.

WO 02/17888

PCT/EP01/09908

- 6 -

To verify this approach fluorescein microparticles were covered with a different number of polyelectrolyte layers. Dyes like fluorescein can be considered as model substances for a large class of drugs. Afterwards core dissolution was initiated by a pH change and monitored by the increasing
5 fluorescence in the bulk.

It was found that polyelectrolyte multilayer shells assembled around cores consisting of low molecular weight compounds provide barrier properties for release under conditions where the core is dissolved. This finding is a
10 novel approach for fabrication of systems with prolonged and controlled release properties. The release can be adjusted with the number of assembled polyelectrolyte layers. The capsule permeability for low molecular weight compounds was found to depend strongly on the number of polyelectrolyte layers in the capsule.

15 A large variety of synthetic polyelectrolytes with different properties, lipids, and polysaccharides have been already used for multilayer assembly [9, 12]. This provides many possibilities to tune the release properties of the shells together with ensuring biocompatibility and possibility of using
20 various cores. The assembling of shells by LbL technique opens new pathways for biotechnological applications, where controlled and sustained release of a substance is required. Many problems connected with drug formulation, release, and delivery, controlling the concentration in the organism and periodicity of its reception can be solved by the formation of
25 shells on precipitates and nanocrystals. Further it is not difficult to add to the polyelectrolyte layer targeting properties. This way the affinity of polyelectrolyte multilayer coated drugs to specific or injured tissues can be increased.

30 In the following the release properties of capsules with regard to the number of layers forming the capsule wall are discussed in more quantitative terms using fluorescein as model substance (MW ~ 350 Da).

WO 02/17888

PCT/EP01/09908

- 7 -

The permeation of a molecule such as fluorescein through the shell wall is described by its permeability (P). Equation (1) combines a flux (J) with parameters of the system and the rate of change of fluorescein concentration, c^s , in the bulk. When this rate is constant one may easily calculate the permeability (P) from the slope of the fluorescence increase (part 2 of Fig.3).

$$\frac{dc^s}{dt} V_0 = J = P \cdot (c^i - c^s) \cdot S \quad (1)$$

where V_0 is the volume of solution, J is the fluorescein flux through the capsule walls with the total surface area of S, and $(c^i - c^s)$ is the difference of the fluorescein concentration inside (c^i) and outside (c^s) the capsules.

As long as a core of solid fluorescein is present within the capsules, the interior of the capsules contains a saturated fluorescein solution, c^s , of 25 mg/ml. Hence, the concentration difference at the beginning of the process in the right side of Eq.1 can be safely replaced by $c^i = c^s$. The capsules were assumed spherical with an average diameter 5 μ m. The permeability can thus be calculated from

$$P = \frac{dc/dt \cdot V_0}{c^i \cdot S} \quad (2)$$

For 8 to 18 layers the permeability value was in the order of 10^{-8} m/s. Assuming single polyelectrolyte layer thickness of 2 nm the permeability can be converted into a diffusion coefficient (D) by means of multiplying the permeability with the shell wall thickness. The calculated diffusion coefficients are in the order of 10^{-18} m²/s.

If the permeability of the polyelectrolyte multilayer is provided by diffusion through the entangled polymer network, it should scale with the inverse of the layer thickness. The behavior of the permeability times thickness as a function of the number of layers is shown in Figure 4. As can be seen, the

WO 02/17888

PCT/EP01/09908

- 8 -

permeability decreases with increasing layer number much faster than expected from a straightforward thickness increase. Only from approximately 8 layers onwards the permeability multiplied by the shell thickness becomes a constant indicating that the permeability is now controlled by the thickness increase, for example, the diffusion limiting region is the polyelectrolyte layer. This finding is consistent with the earlier observations [11] where it was shown that the conformation of the first eight layers differs from that of further assembled layers. These deeper layers are more denser resulting in a fivefold reduction of the estimated diffusion coefficient as can be inferred in Fig. 4.

A permeability coefficient might also be calculated from the release profile at the third stage of the fluorescein release curve. It can be expected that the time dependence of the release at this stage is exponential:

$$c^e(t) \sim \left(1 - e^{-\frac{P}{V} t} \right), \quad (3)$$

where $\bar{S}$ and V represent the surface and the volume of an average capsule. However, the permeability estimated from this equation was one order less than the one calculated from the slope of the linear region. There are currently two possible explanations for this discrepancy. Either the polydispersity of the particles contributes to an apparent prolonged release, because the characteristic time release $V/\bar{S}P$ increases linearly with the particle radius. Or, the relaxation of the osmotic stress towards the end of release may reduce the permeability.

In the following the permeation mechanism is discussed in more detail. One may distinguish between diffusion through water filled pores and a "bulk" diffusion mechanism through homogeneous phase of polyelectrolyte multilayer shell. For thinner walls the drastic dependence on shell thickness

WO 02/17888

PCT/EP01/09908

- 9 -

may be explained either by existence of pores that are successively closed by further layer deposition or by thickness dependent diffusion coefficient. The latter was indeed observed with permeation studied by planar polyelectrolyte films where typical values of D between 10^{-18} and 10^{-20} m²/sec were derived [11]. In order to compare our results with those of the previous study we should remark the following differences.

- According to the method described in [11] the films were prepared by drying after each adsorption step which is not possible during particle coating and which leads to a denser film.

- The measurement consisted in depositing a dye probe in a defined depth into the film and observing time dependent fluorescence changes due to quencher diffusion. For sufficiently small pore concentration, which is surely the case here, this technique is only sensitive to "bulk" diffusion and will not reflect the permeation through pores.

Thus one may expect that the diffusion coefficients derived from permeability data are larger than values measured in [11]. Still, this cannot explain the difference of 3-4 orders of magnitude and therefore they more probably correspond to diffusion through pores. Comparing the diffusion coefficient D with that in bulk water ($\sim 10^{-10}$ m²/s) which is an upper limit for the diffusion in the pore volume we may estimate the fraction of pore volume inside the walls larger than 10^{-5} .

Next a possible mechanism of pore formation is addressed. A destabilization of the wall may be expected, since on increasing the pH towards 8 the aminogroups of PAH may deprotonate and this may result in pore formation within the multilayers. Another possibility of pore formation is the osmotic pressure due to the water coming to the interior as a result of fluorescein core dissolution. The related hydrostatic pressure difference creates a tension in the wall which may widen existing or create new

WO 02/17888

PCT/EP01/09908

- 10 -

pores. As seen on Fig.3 (part 1), especially for bigger numbers of layers, in the beginning of dissolution the release is sustained. It is assumed that the polyelectrolyte shell suffering osmotic pressure from inside resists release of fluorescein until the fluorescein molecules develop a path out of capsules. The pores are formed as a result of this rearrangement of polyelectrolyte multilayers. It should be noted that templates and the resulting capsules have different diameters and also heterogeneous wall thicknesses. The diffusion coefficient is thus an average but there may still be larger templates with slower release profile.

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In summary, it can be noted that by increasing the number of layers of polyelectrolyte capsule walls the permeability of the capsules can be reduced. For a delayed release preferably capsules having ≥ 8 , more preferably ≥ 9 and most preferably ≥ 10 layers are used.

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In a preferred embodiment the invention relates to a novel approach for encapsulating materials and molecules, such as macromolecules, biopolymers, drugs etc., in pre-formed hollow polyelectrolyte capsules. According to this embodiment of the invention the material or/and molecules can be introduced in hollow capsules rather than forming capsules around the materials/molecules.

20

The properties and structure of polyelectrolyte multilayers are found to be sensitive to a variety of physical and chemical conditions of the surrounding media. In particular, the pH is one of the physico-chemical parameters, which influences the state of the inter-polyelectrolyte complex [20,21], especially in the case, if the charge of one polyelectrolyte in the complex depends on the pH. The polyelectrolyte pair, poly(styrene sulfonate) (PSS) and the relatively weak polycation poly(allylamine hydrochloride) (PAH), has been most extensively used for producing multilayer films on flat and colloidal surfaces (1) and serves as exemplary

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WO 02/17888

PCT/EP01/09908

- 11 -

polyelectrolyte pair for illustrating the invention. However, the invention is not limited to this specific polyelectrolyte pair.

By using different polyelectrolytes, the permeability can be further modified. The combination of cationic and anionic polymers results in almost unlimited variation possibilities. Characteristic values, such as polarity and polymeric rigidity can be adjusted via the chemical composition and have a specific effect on different substance classes. For example a reduction of the polarity results in a higher hydrophobicity, which decreases the permeability for polar, water soluble substances, whereas the permeability for unpolar, oil soluble substances is enhanced.

In addition, the permeability behaviour of the capsules can be strongly modified via the molecular weight and the degree of branching of the polyelectrolytes used. The molecular weight is advantageously set between 10 000 and 500 000 g/mol, wherein generally higher molecular weights and increasing branching lead to higher capsule stability and lower permeability.

By varying the pH of the medium, the permeability of the walls of microcapsules can be varied. While it is not intended to be bound to a specific mechanism of the pH-induced permeability change, it is supposed that changes of the polyelectrolyte charge upon pH variation are able to induce pore formation (22) or loosen the polyelectrolyte network, thus enabling polymer penetration. An influence of the ion concentration going along with the pH change may also contribute to the permeability change.

This possibility of switching the capsule walls between an open and closed state provides a convenient and efficient tool to control the uptake and release of molecules, in particular polymers, biopolymers and nanoparticles. For instance, the capsules might be loaded at low pH and after increasing the pH the material is captured inside. For release the pH can be slightly

WO 02/17888

PCT/EP01/09908

- 12 -

decreased again, whereby the kinetics of release can be tuned by the pH. The herein demonstrated possibility of controlling loading and release of macromolecules into and from polyelectrolyte capsules allows for widespread application. The described pH-induced permeability change of the polyelectrolyte network in the film is a general mechanism for modifying the permeability of polyelectrolyte multilayers and is not limited to a specific composition of the shell. Investigations on flat polyelectrolyte films made from weak polyelectrolytes have shown that pores can be created by changing the pH or the salt concentration.

We have found that permeability of capsules composed of polyelectrolytes, in particular of weak polyelectrolytes, strongly depends on the pH. Being impermeable for macromolecules at a pH when the charges of two polyelectrolytes forming the capsule wall are compensated, capsules are open at a pH, at which a weak polyelectrolyte is completely charged. This dependence gives an opportunity to encapsulate polymers by opening and closing a capsule by means of pH. By means of AFM at the open state pores ranging in size from 100 to 300 nm were observed in the capsule wall. A further remarkable feature of the process of pore formation by pH variations is that the pore formation or increased permeability is reversible.

Further permeability of the capsules inversely depends on the number of layers they consist of. Employment of both these factors gives a possibility to regulate capsule release properties for low as well as for high molecular weight compounds. Fluorescein particles, stable at acidic pH can be used as core. They can be covered with different numbers of polyelectrolyte layers. The kinetics of fluorescein dissolving and release was measured at base pH. It was found that time of core decomposition depends strongly on the capsule wall thickness. During the dissolving a sustained release of fluorescein was observed.

WO 02/17888

PCT/EP01/09908

- 13 -

Permeability control by means of pH and capsule wall thickness is proposed as a new approach for controlled uptake and release of different substances into and from capsules. It opens avenues for using polyelectrolyte capsules for many applications, such as drug carriers, microreactors and so on.

In summary, it can be stated that by increasing the pH a reduced capsule permeability can be achieved and by reducing the pH an increased capsule permeability can be achieved, whereby this process is reversible. It was found that capsule walls particularly at a pH of > 8 , more preferably at a pH of > 10 are impermeable (closed condition), whereas at a pH of < 6 , particularly of < 4 a permeability (open condition) of the capsule walls can be achieved. The change in pH represents a simple way for adjusting the permeability and by amending the surrounding medium makes it possible to provide for a permeability switch for the capsules.

This application also presents a novel, simple and very gentle method for targeted encapsulation and release of sensitive, macromolecules by controlling the capsule wall permeability by the salt concentration. Salts have an immense importance in the living organisms. The salt concentration in the cytoplasm and in the extra cellular medium is strongly regulated by the permeability of the cell membranes, by osmosis and by active transport via energy driven pumps. The physiological concentration of sodium chloride in plasma is about 0.15 M, other ions, such as potassium or calcium are crucial for signal transduction across the membrane and inside the cell.

The permeability of hollow polyelectrolyte capsules, preferably of capsules made from PAH/PSS can be reversibly switched between an open and closed state by varying the salt concentration in a narrow concentration range between $0.5 \dots 2 \times 10^{-2}$ M/l. The increase of the salt concentration

WO 02/17888

PCT/EP01/09908

- 14 -

leads to a reversible opening of the capsule wall for large molecules of MW above 50 000 Da, preferably above 70 000 Da.

5 The absence of pores in scanning force microscopy images, the relatively small permeability and the step-like decrease of the Förster resonance energy transfer in the wall with increasing salt concentration indicates a weakening of the electrostatic interactions between the polyelectrolytes and a subsequent swelling connected with a much better diffusion of the polymers. Salt triggered permeability is a very easy, important, and
10 effective way for the encapsulation and release of, especially, sensitive macromolecules such as enzymes, proteins, or DNA in micro- and nanocontainers.

In summary, it can be noted that by increasing the salt concentration an
15 increase of the permeability of the capsule walls can be achieved, whereas reducing the salt concentration causes a reduction of the permeability (closed condition). Preferably the capsule walls are made permeable by a salt concentration of at least 2×10^{-2} M, more preferably of at least 3×10^{-2} M and most preferably of at least 1×10^{-1} M. If a closed condition of
20 the capsules is desired, the surrounding conditions are adjusted to a salt concentration of at most 5×10^{-3} M, more preferably of at most 1×10^{-3} M and most preferably of at most 1×10^{-4} M.

Suitable salts according to the invention are all heteropolar compounds
25 comprising at least one anion and at least one cation. The anions and cations can be singly charged or multiply charged ions. For adjusting the permeability preferably inorganic salts are used, particularly metal salts, such as metal halogenides, particularly alkali metal halogenides. Examples of particularly preferred salts are salts containing an alkali metal cation,
30 particularly Li^+ , Na^+ , K^+ , an earth alkali metal cation, particularly Mg^{2+} , Ca^{2+} or a different metal cation, e.g. Al^{3+} , an iron ion, etc. and an anion, e.g. a halogenide anion, or a different anorganic anion. The metals may

WO 02/17888

PCT/EP01/09908

- 15 -

also be replaced by other positively charged groups, e.g. an ammonium group, a sulfonium group or a phosphonium group. However, it is also possible to use organic salts, particularly salts containing organic anions.

5 Apart from the salt concentration, the permeability can also be adjusted by the ion concentration and ion composition of the environment, respectively, wherein also here it was detected that for higher ion concentrations a permeability is achieved, whereas for low ion concentrations an impermeable capsule wall was obtained.

10

The ion composition particularly plays a role, since the permeability of the capsules is influenced by interactions between ions of the surrounding medium and the charges of the polyelectrolyte shells. By suitably selecting the ions of the surrounding medium, e.g. ions forming complexes with the polyelectrolytes of the capsule shells, the permeability can be adjusted.

15

The dependence of permeability on salt concentration, ion composition and ion concentration can be particularly used advantageously by administering encapsulated active substances to organisms, e.g. mammals and particularly humans. Therefore capsules can first be charged with active substances, wherein in vitro the capsule walls are made permeable to the active substance with the help of the above described measures. Then the encapsulated active substances can be stored in a stable form, e.g. by adjusting a salt concentration of the storage medium to $< 5 \times 10^{-3}$ M. This can be done e.g. by storage in distilled, preferably in sterile distilled water. The administration of charged capsules is accompanied by an amendment of the surrounding medium, wherein, as already stated above, the physiological concentration of sodium chloride in plasma is about 0.15 M. Under these surrounding conditions the capsules are permeable to the active substance so that the active substance is released in the target tissue.

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WO 02/17888

PCT/EP01/09908

- 16 -

The invention also relates to a simple method for the polyelectrolyte capsules allowing opening and closing of the wall for large polymers but also for small organic molecules and to control the permeability in a simple way by temperature treatment.

5 It has been shown, that the permeability of thin polyelectrolyte walls can be switched easily by heat. This is probably caused by an temporarily swelling of the wall due to stronger dissociation of the cation-anion bonds at higher temperature, followed by hydration of the formed charges and
10 entanglement of the polymer chains. The reversibility of this process provides an excellent tool for loading hollow polyelectrolyte capsules with large macromolecules, polymers or nanoparticles for drug encapsulation as well as for the controlled and intelligent release of active substances. For example, in the agriculture many pests are active mainly at high
15 temperatures. Encapsulation of pesticides in such capsules can ensure a release only at hot and sunny days. Or, in the new generation of intelligent clothes, deodorants will be released only in case of strong wetting.

Preferably at a temperature of up to 50°C, more preferably up to 40°C,
20 the permeability is low (closed condition), whereas at a temperature of above 50°C, particularly of at least 60°C, permeable capsules (open condition) are obtained. By increasing the temperature in a simple way a permeability increase can be achieved.

25 In a further embodiment of the invention the permeability of polyelectrolyte multilayer capsules is amended by varying the solvent composition or solvent concentration. Thus, the release behaviour can be adjusted via the solution, wherein as solution e.g. polar solutions, particularly water or/and polar solutions or mixtures thereof can be used. Particularly suitable
30 solutions for adjusting the release behaviour are alcohol/water mixtures, wherein the alcohol content, e.g. methanol, ethanol or propanol, can be adapted to the individual application.

WO 02/17888

PCT/EP01/09908

- 17 -

Further, the permeability of polyelectrolyte multilayer capsules can further be influenced by adding surfactants, such as sodium dodecylsulfate (SDS) or dipalmitoyl-DL- α -phosphatidyl choline (DPPC) to the surrounding medium or as coating for the core used in capsule production.

5 The permeability of polyelectrolyte multilayer capsules can also be influenced by light. Photosensitive groups in the electrolyte layer, such as azo compounds or organic bisazides can be modified chemically by light, which may lead e.g. to an increase of the permeability or to a reduction of
10 the permeability, if the photosensitive compositions are selected which cross-link by exposure to light.

As explained above, according to the invention it is possible to insert further components into the polyelectrolyte layers in order to obtain a
15 broad modification of the permeability. The further components can e.g. be bound covalently to the polyelectrolyte or be independent molecules or particles, which are only embedded in the polyelectrolyte layers. The further components can be incorporated together with the polyelectrolyte or as independent units by using various methods, preferably the layer-by-
20 layer method, but also a single step method or subsequent diffusion.

The microcapsules, in particular hollow microcapsules, whose permeability can be controlled according to the invention preferably can be fabricated by alternating deposition of oppositely charged polyelectrolytes on soluble
25 colloidal templates. The polyelectrolyte capsules can e.g. be prepared by covering templates, ranging e.g. from 60 nm, preferably from 100 nm, to 10 μm , preferably to 2 μm in size, with alternating layers of polycations and polyanions [6,7]. After formation of a polyelectrolyte wall of sufficient thickness, the templates can be dissolved and hollow capsules are
30 obtained. The templates can e.g. be removed by changing the pH or by oxidative decomposition of the template. This is possible due to the remarkable property of the capsule wall to be permeable for small

WO 02/17888

PCT/EP01/09908

- 18 -

molecules having a molecular weight of less than 10 000, preferably less than 5 000 and particularly less than 1 000 but not for polymers. The template determines size and shape of the capsule. Monodisperse capsules in the range from 500 nm to 10 μ m can be prepared. Suitable capsules and their production and composition are described, e.g. in WO99/47252, WO99/47253, WO00/03797 and WO00/77281, the disclosure of which is herein incorporated by reference.

A remarkable feature of the capsule wall is that their properties can be tuned within wide ranges by varying the layer material and/or number or/and by varying environmental conditions such as pH, salt concentration, ion strength, ion composition, solvent concentration, solvent composition or/and temperature. These capsules offer broad perspectives in nanoscale encapsulation of drugs, enzymes, DNA, minerals, dyes, polymers, proteins and/or other active macromolecules.

The capsules are preferably fabricated by alternate adsorption of oppositely charged polyelectrolytes onto the surface of colloidal particles. Different cores having a size varying from 0.06 preferably from 0.1 to 10 μ m, such as inorganic colloidal particles, biological cells, protein aggregates, drug nanocrystals can be used. Hollow capsules can be produced by subsequently dissolving the core.

The permeability of capsules is of essential interest because of its significant importance in diverse areas, relating e.g. to biotechnology, medicine, food industry, etc.

The invention shall be further illustrated by the following examples and figures:

Fig. 1: Scheme of the polyelectrolyte multilayer deposition process and of the subsequent core dissolution. The initial steps (A-D) involve stepwise

WO 02/17888

PCT/EP01/09908

- 19 -

shell formation on a fluorescein core. After the desired number of polyelectrolyte layers is deposited the coated particles are exposed to pH 8 (E) and core dissolution with fluorescein penetration into the bulk is initiated resulting finally in fully dissolved cores and remaining empty capsules (F).

Fig. 2: Fluorescence increase upon time, obtained by dissolving fluorescein particles covered with shells of different thickness (9, 13, 15, and 18 layers).

Fig. 3: Three stages of fluorescein core dissolution covered with 17 PSS/PAH layers.

Fig. 4: Fluorescein diffusion as a function of layer number.

Fig. 5: Permeation and encapsulation of FITC-dextran (M.w. 75 000) into polyelectrolyte multilayer capsules. Left - pH = 10, center - pH = 3, right - pH increased to 10 after the capsules were loaded with FITC-dextran at pH = 3. The bulk FITC-dextran was removed by washings at pH = 10. Top - scheme, center - confocal images of the capsules, bottom - fluorescence profile along the line indicated in the confocal images.

Fig. 6: Confocal Laser Scanning Microscopy images showing: a) capsules for which the washings and the core dissolution was performed in the presence of 0.05 M NaCl; b) capsules which were washed with pure water after each deposition step; 24 h incubation in a 10^{-3} M PAH-Rho solution; c) same procedure as in b) but incubation in presence of 10^{-2} M salt.

Fig. 7: Efficiency of the Förster Resonance Energy Transfer in capsules containing the 10th layer PAH-fluo and the 12th layer PAH-rho in dependence on the salt concentration. The inset shows a typical fluorescence spectrum of these capsules. Excitation was set at 495 nm.

WO 02/17888

PCT/EP01/09908

- 20 -

Fig. 8: Time-dependent decrease of the FRET efficiency as a function of the salt concentration.

Fig. 9a): Capsules, loaded with 5×10^{-3} M PAH-rho according to the protocol shown in Fig. 9b) Same capsules after release of the encapsulated PAH-rho induced by a 3×10^{-2} M salt solution.

Fig. 10: Scheme of encapsulation and release of macromolecules by switching the permeability salt concentrations changes.

Fig. 11a): CLSM image of capsules in presence of 10^{-3} M Fluorescein-PAH (MW 70 000 g/mol, polymer exclusion); b) CLSM image of capsules in presence of 10^{-3} M Rhodamine-Dextran (MW 50 000 g/mol, accumulation);

Fig. 12a): CLSM image of capsules in presence of 10^{-3} M Rhodamin-PAH (MW 70 000 g/mol) at 23°C ; b) encapsulated Rhodamine-PAH in capsules after heating the solution for 20 min to 60°C and subsequent washing with water.

Fig. 13: Permeability of capsules composed of 8 layers of PSS-PAH for a labeled dextran, molecular weight 77 000 at a low and high pH.

Fig. 14: Characteristic time of dissolving of fluorescein core, covered with different number of PSS-PAH polyelectrolyte layers.

Fig. 15: The permeation of high molecular weight molecules.

Table 1: Percentage of filled capsules in solutions of PAH-rho, 5×10^{-3} M, after 24 hours incubation time and in presence of different salt concentrations.

WO 02/17888

PCT/EP01/09908

- 21 -

Examples

Example 1: Varying number of polyelectrolyte layers

5 Materials

Polyelectrolytes. Sodium poly(styrene sulfonate) (Na-PSS, MW ~70 000), poly(allylamine hydrochloride) (PAH, MW ~50 000), and fluorescein, sodium salt, were obtained from Aldrich. Ethanol, sodium chloride, boric acid and hydrochloric acid were purchased from Sigma. All materials were used
10 without further purification.

The water used in all experiments was prepared in a three stage Millipore Milli-Q Plus 185 purification system and had a resistivity higher than 18.2 MΩ cm.
15

Methods

Fluorescein particles were prepared by addition of one part of ethanol and four parts of hydrochloric acid, pH 2, to one part of 15 mg/ml aqueous fluorescein solution. The particle size was measured by optical microscopy.

20 The 4-9 μm in diameter fluorescein particles were washed in hydrochloric acid by 5 repeated centrifugation circles at 450 g. To prevent preliminary core decomposition all further multilayer deposition and washings were performed in hydrochloric acid at pH 2.

25 Polyelectrolyte multilayer assembly. The multilayer assembly was accomplished by adsorption of polyelectrolytes at a monomer concentration of 10^{-2} M in 0.5 M NaCl, pH 2. Oppositely charged polyelectrolyte species were subsequently added to the suspension of fluorescein particles followed by repeated centrifugation cycles in
30 hydrochloric acid [7]. Fluorescein particles were allowed to interact with polyelectrolyte solution for 15 minutes. Fluorescein particles were

WO 02/17888

PCT/EP01/09908

- 22 -

centrifuged at 700 g for 10 minutes. Gentle shaking followed by 1 minute ultrasonication was used to disperse particles after centrifugation.

Fluorescence spectroscopy. The core dissolving was conducted in $\text{H}_3\text{BO}_3\text{-NaCl-NaOH}$ buffer, pH 8. The kinetics was followed by recording the time dependence of the emission at 522 nm. Excitation was set at 488 nm.

Confocal microscopy. Confocal images of capsules after dissolving the core were obtained by means of a Leica confocal scanning system. A 100 x oil immersion objective with a numerical aperture of 1.4 was used.

Figure 1 provides the scheme of fluorescein particles encapsulation and release. After LbL adsorption (Fig. 1A-1D) core dissolution is initiated by changing the pH from pH 2 to pH 8 (Fig. 1E) and completed after a certain period of time (Fig. 1F).

Fluorescein particles rapidly dissolve at pH 8. The idea was thus to slow down the rate of core dissolving by covering the particles with a polyelectrolyte multilayer. Shells walls consisting of a different number of layers were fabricated and examined with regard to their fluorescein permeability behavior. Fluorescence spectroscopy is a convenient tool for the determination of the core dissolving rate because the fluorescence of the core is completely suppressed as a consequence of the self-quenching of the dye. Upon releasing the dye into the bulk the fluorescence intensity increases. Thus the rate of cores dissolving can be directly followed by measuring the fluorescence increase in the sample.

In Fig. 2 typical time-dependent fluorescence curves obtained by switching the pH to 8 are shown. Fluorescein particles covered by layers of different thickness (9, 13, 15, and 18 layers) are compared with the control demonstrating the dissolving of naked fluorescein particles.

WO 02/17888

PCT/EP01/09908

- 23 -

As shown in Figure 3, after a comparatively short induction period (1) the rate of dissolving becomes constant (2) before finally the fluorescence in the bulk levels off (3). The initially more slowly increasing fluorescence is related to the start of core dissolving. At this stage of the process the structure of the polyelectrolyte multilayer may change because of the nascent osmotic pressure coming from dissolved fluorescein molecules. Shortly after the beginning of core dissolution the concentration of fluorescein inside the capsules becomes constant and almost saturated, since a steady state situation between progressing core dissolution and permeation is established. One may further assume a constant concentration gradient between the shell interior and the bulk because the bulk solution can be assumed as being infinitely diluted. Therefore the rate of fluorescein penetration through the polyelectrolyte layers to the bulk becomes constant. Indeed, a linear increase of the fluorescence is observed (2). This state corresponds to the stage of dissolution depicted in Figure 1E. The slope of the linear region decreases with the number of polyelectrolyte layers. Obviously an increasing number of adsorbed layers reduces the fluorescein penetration. After the core is completely dissolved, the fluorescein concentration inside the shell equilibrates with the bulk. The driving force for diffusion decreases and the release levels off (3).

Figure 4 shows the behaviour of the permeability times thickness as a function of the numbers of layers.

Example 2: Permeability as function of pH

Sodium poly(styrene sulfonate) (Na-PSS, MW ~ 70 000), poly(allylamine hydrochloride) (PAH, MW ~ 50 000), dextran (MW ~ 75 000 and 4 000 000), and bovine serum albumin all labeled with fluorescein isothiocyanate (FITC) were obtained from Aldrich. Hollow polyelectrolyte capsules were fabricated at pH=7 by alternating adsorption of 8 layers PSS/PAH onto MF-particles of a diameter of 5.2 μm (microparticles GmbH, Berlin), using

WO 02/17888

PCT/EP01/09908

- 24 -

the filtration protocol (15). The cores were dissolved at pH = 1. Washings in 50 mM NaCl followed. Confocal images were taken by means of a confocal laser scanning microscope (TCS Leica). The excitation wavelength was 488 nm. The capsules were suspended into the FITC labeled polymer solution of a concentration of 1 mg/ml.

The exclusion properties of hollow polyelectrolyte capsules composed of 8 PSS/PAH layers templated on 5.2 μ m melamin formaldehyde (MF) - particles for FITC-labeled dextran (with MW about 75 000) have been studied as a function of pH. Fig.5 (left) provides a confocal image of capsules in the presence of FITC-dextran at pH = 10. The interior of the capsules remains dark, while the background is fluorescent. This proves that at this condition the capsule wall is not permeable for FITC-dextran. Even deformed capsules do not reveal any fluorescence inside. However, at pH = 3 the capsule interior becomes as fluorescent as the bulk (Fig.5, center). This can only be explained by opening of the capsules for FITC-dextran at this low pH value.

It has to be mentioned that the "open" and "closed" states at relatively low pH and high pH, respectively, were observed for more than 90% of the capsules. The open state for FITC-dextran was observed for pH values up to 6. From pH 8 onwards most of the capsules are closed. At a pH value in between, i.e. pH = 7, open and closed capsules were simultaneously present.

The possibility of loading is demonstrated in Fig.5 (right) where the capsules were initially exposed to a FITC-dextran solution at pH = 3. Then the pH was shifted to 10 and the rest of FITC-dextran was removed from the bulk by centrifugation. It is remarkable that the capsules remain filled with fluorescent material as shown by the fluorescence profile through the confocal image. The interior of the capsule observes a bright and constant over time fluorescence while there is no fluorescence signal from solution.

WO 02/17888

PCT/EP01/09908

- 25 -

Similar experiments of changing the capsule wall permeability by pH changes and subsequent capsule loading were performed with FITC-dextran of a MW of 2 000 000 and FITC-labeled bovine serum albumin. The results are analogous to those with FITC-dextran of MW 75 000, but the pH value of the transition between the closed and open state differs by 1-1.5 pH units.

Example 3: Controlling capsule wall permeability by the salt concentration

Two different types of polyelectrolyte capsules were used for these permeability experiments. 8 layers of sodium polystyrenesulfonate PSS and polyallylamine PAH were adsorbed on monodisperse templates of diameter 4.7 μm consisting of weakly polymerized melamine-formaldehyde resin. In one batch, core dissolution was conducted at pH 1 and the subsequent washings were performed in the presence of 0.05 M NaCl. This protocol yielded crumpled capsules (Fig. 6a). These capsules were covered by two additional layers and subsequently washed with Millipore water ($\sigma < 18 \text{ M}\Omega \text{ cm}$). After this treatment, the capsules assumed a spherical shape.

The cores in the second batch were dissolved in 0.1 M HCl and washed afterwards with Millipore water thus avoiding the addition of salt. The latter protocol yielded homogeneous population of capsules with a spherical shape (Fig. 6b). The permeability behavior of the two types of capsules was similar. Essentially the results obtained with the second type of capsules will be reported in this example. Only in case of remarkable differences results obtained with capsules having additional two layers will be mentioned.

The permeability of the capsule walls was investigated by means of fluorescence labeled polymers polystyrenesulfonate PSS (120 000 g/mol), polyallylamine PAH (70 000 g/mol), dextrane (55 000 g/mol) and human albumine (70 000 g/mol). Polyallylamine PAH was used which was labeled

WO 02/17888

PCT/EP01/09908

- 26 -

at every 245th position with rhodamine B. In the Förster resonance energy transfer measurements, polyallylamine PAH labeled at every 120th position with fluoresceine was used. The capsules were added to a 5×10^{-3} M solution of the respective probe polymers, which concentration is always expressed in monomer units. The polymer permeation was followed by means of confocal imaging (TCSCN Leica, Germany). The amount of fluorescent molecules inside and outside the capsules was quantified.

The image in Fig.6b shows a confocal scan through the equatorial plane of the capsules, which were incubated with the labeled polymer solution for 24 hours. About 90% of the capsules excluded the polymers. The few with polymer filled capsules were broken and observed large holes through which the polymers could have diffused almost instantaneously into the capsule interior.

A similar experiment was then performed in the presence of 0.1 M NaCl. The confocal image (Fig. 6c) shows, that the interior of all capsules contains approximately the same concentration of the polymer as present in the bulk. This finding is consistent with an increase of the permeability of the polyelectrolyte wall for polymers in the presence of salt. This "wall opening" was observed for positively and negatively charged polymers as well as for the dextrane representing a neutral polymer. Hence, salt induced changes in the structure of the probe polyelectrolytes, such as coiling of the charged species cannot be the responsible mechanism for the penetration. Rather salt-induced changes of the structure and properties of the polyelectrolyte complexes in the wall have to be considered as the cause for the strong increase in permeability. As evident from the images, the fluorescent probe molecules were accumulated on the surface or inside the capsule wall. This was expected for PSS, which is adsorbed onto the positively charged capsule surface, but either for the labeled PAH an exchange of PAH molecules in the capsule wall or an adsorption to the inner PSS layer has to be assumed.

WO 02/17888

PCT/EP01/09908

- 27 -

The permeability was next investigated in dependence on the salt concentration. The capsules were mixed with 5×10^{-3} M PAH-Rhodamine and increasing concentrations of sodium chloride. The dispersions were incubated for 10 min and the amount of polymer in the interior was compared with the polymer concentration in the bulk. Up to 5×10^{-3} M NaCl, no polymer penetration into the capsules was detected, but for higher concentrations, the capsules became increasingly permeable for PAH-Rh. At 2×10^{-2} M NaCl, the time of PAH-Rh penetration was estimated by means of confocal imaging overtime. The time between salt addition and complete equilibration of the polymer concentration inside and outside the capsules was app. 6 min for capsules consisting of 8 layers. A slower penetration occurring within 20 min was measured for capsules of 10 layers. Confocal imaging could not follow the kinetics at higher concentrations, because the diffusion and equilibration of the polymer concentration was too fast.

The minimum amount of salt for inducing the polymer penetration was determined by incubating the capsules in polymer solution, at various NaCl concentrations for 24 hours. Then, the percentage of filled capsules was determined by counting in the confocal images the number of filled and empty capsules for app. 100 capsules each (Table 1). While in the 10^{-2} M salt solution all capsules contained labeled polymer, in the 0.5×10^{-2} M salt solution only 16% of the capsules appeared to be filled within 24 h. In still lower NaCl, the capsules remained impermeable for the polymer. Hence, the permeability increase of the capsules for macromolecules occurred within a rather narrow range of salt concentration between 0.5 and 2×10^{-2} M.

It was further examined, whether the rate limiting step of permeation after the addition of salt is provided by salt-induced structure changes in the capsule wall making the wall more permeable or by the diffusion of the polymer through the layer. For this the permeation of PAH $M_n = 70\ 000$

WO 02/17888

PCT/EP01/09908

- 28 -

was observed either immediately after the simultaneous addition of 10^{-2} M salt (NaCl) and 5×10^{-3} M PAH or the sample was incubated for 12 hours in 10^{-2} M NaCl and the PAH was added only then. In the second case, it was found that the characteristic permeation time was about 30 min, which was smaller than the characteristic permeation time of about 50 min observed when NaCl and the polymer were added at the same time. These numbers are two fold remarkable. They demonstrate that 1) the permeation of the polymer under the observed conditions is overall slow requiring at least 30 min for concentration equilibration, and 2) that the permeability induction by salt itself requires several minutes.

Regarding the mechanism of the polymer permeation through the capsule wall two extreme situations may be distinguished. Either incubation in the salt solution led to the formation of water-filled pores through the wall across which the polymers can diffuse into and out of the capsule, or the presence of ions in the solution weakens the electrostatic binding between PSS and PAH allowing for diffusion of the polymer through the network via transient bond breaking and re-establishing. Pore formation either induced by pH or by salt was observed for macroscopic flat films composed of weak polyelectrolytes. These relatively large pores had a radius in the order of 10^2 nm. SFM was thus applied to polyelectrolyte capsules either prepared by drying a capsule dispersion in H_2O or in 0.1 M NaCl applied onto a mica substrate. However, except the presence of some salt crystals no differences in the capsule wall topology were observed. This result argued the formation of large pores as the cause of the permeability increase.

Assuming that the polymer penetration occurs via diffusion through water-filled pores the apparent pore cross section is of the order of 1000 nm^2 . This would compare to either one pore of 17 nm radius or to 80 pores of 2 nm each. In any case, it is worth to note that the pore area would constitute a very tiny area fraction of the capsule surface only. From these

WO 02/17888

PCT/EP01/09908

- 29 -

considerations, it becomes clear that with SFM it is hardly possible to detect these pores even if their existence is sure.

In order to find changes in the capsule wall morphology in dependence on the salt concentration of the bulk electrolyte, Förster resonance energy transfer FRET between fluorescent labeled polyelectrolytes was applied. The capsules were covered by addition of six polyelectrolyte layers in the order PSS, PAH-fluoresceine, PSS, PAH-rhodamine, PSS, PAH. The polymers were adsorbed in presence of 0.5 M NaCl, but afterwards the capsules were extensively washed by water. FRET of the capsule dispersion was measured 5 min after adding NaCl solutions of different concentrations. Excitation was set at 495 nm, where only fluoresceine absorbs light. If rhodamine molecules are located near the fluoresceine, (up to 6 nm) resonance energy transfer takes place and one can observe the rhodamine fluorescence, too (Fig. 7, inset).

The relative transfer efficiency E_{FRET} was defined as $E_{\text{FRET}} = (I_h - 0.4I_n) / (0.6I_n + I_h)$, where I_n is the fluorescence intensity at 522 nm corresponding to the maximum in fluoresceine emission and I_h is the intensity at 582 nm, corresponding to the rhodamine emission.

The factor 0.4 takes into account the fluorescence of the donor at the acceptor emission wavelength. The plot of E_{FRET} against salt concentration shows a clear decrease in the transfer efficiency (Fig. 7), occurring exactly in the concentration range where the capsule wall becomes permeable for PAH. At higher salt concentrations, the E_{FRET} kept almost constant. Hence, it can be concluded that FRET reported remarkable changes in the topology of the polyelectrolyte wall occurring within a narrow salt concentration range around 10^{-2} mol/L. The decrease of E_{FRET} with the salt concentration indicates an increase of the distance between the dye molecules of rhodamine and fluoresceine. The FRET signal was completely restored within 5 min when the capsules were expose to water again. These

WO 02/17888

PCT/EP01/09908

- 30 -

findings are obviously consistent with some reversible swelling of the polyelectrolyte layer.

The correlation between the permeability increase of the wall opening and FRET decrease allowed for characterizing the salt-induced capsule wall changes in the absence of a permeating polymer. The latter may have had some influence on the wall properties, especially as much remarkable amounts of the permeating polymer adsorbed to the wall. The capsules were introduced in 1.5×10^{-2} M NaCl and the E_{FRET} was measured in dependence on the time (Fig. 8). After 20 min the FRET was saturated at a value of $E_{\text{FRET}} = 0.31$. The time scale of the changes was consistent with the permeability change observed by means of confocal microscopy. Both, the time and the degree of the FRET signal change related to wall structure changes increased with the salt concentration.

The mechanism and driving forces causing both layer permeability increase and the decrease of the fluorescence energy transfer occurring in a narrow salt concentration range are discussed below. The rather slow permeation indicates, if water-filled pores are assumed as the basic pathway for permeation, a very small pore area. On the other hand, the change of the FRET signal can only be understood assuming conformational changes affecting the majority of the labeled polymers. Hence, salt induces layer changes throughout. This is less constant with assuming the much-localized formation of a small number of pores. Therefore we interpret the permeability increase as a result of an increased solubility or interactions of the permeability species with the layer polymers, which are caused by an increased salt concentration.

A higher salt concentration was found to soften the structure of the layer because the ion pairs found between the polyanions and the polycations may partly open, because the free ions available in the bulk would screen the charges in the layer. Layer swelling can thus be understood as a

WO 02/17888

PCT/EP01/09908

- 31 -

molecular elasticity relaxation of the polymer pairs, leading to a more bulky arrangement of the layer polymers. It is worth to note that at 10^{-2} M NaCl the Debye screening length is just 3 nm comparing well to a layer thickness increment of a polyanion/polycation pair. At lower electrolyte concentrations, the electrostatic interactions have a larger range providing an electrostatic interaction over the whole layer.

An important issue for many applications is the reversibility of the permeability switching process. The fact, that the capsules were prepared at high salt concentrations and are nevertheless impermeable for the macromolecules after washing demonstrates the reversibility of the capsule opening and closing. In further experiments the time of closing the capsule wall was determined. A 3×10^{-2} M NaCl solution was applied for 30 min to the capsules. Afterwards they were diluted into pure water to app. 5×10^{-3} M NaCl. After ten minutes, the capsules became impermeable for the PAH-Rhodamine as was observed by means of confocal imaging. The FRET signal was recovered within 5 min (Fig. 8).

The reversibility of the wall permeability change for polyelectrolytes allows for an easy and soft encapsulation of macromolecules, such as polypeptides, DNA, enzymes, or polymers avoiding any chemical stress caused by aggressive substances, solvents, pH, or heat applied during encapsulation. However, loading of capsules with macromolecules at high salt concentration following by washing with water yielded only small amounts of encapsulated polymer in the interior. Probably, the macromolecules were washed out faster than the wall closed.

A much higher efficiency of loading was however achieved when the polymer for encapsulation was present in the bulk during closure of the wall induced by salt removal. (Fig. 10). By this way, the capsules were loaded with a 5×10^{-3} M PAH-Rh solution (Fig. 9). Taking the fluorescence intensity of the confocal image of the PAH-Rh solution as standard, the

WO 02/17888

PCT/EP01/09908

- 32 -

amount of encapsulated polymer could be determined. The average of the interior PAH-rho concentration was about 50% of that of the initial solution.

5 Another important property of the loaded capsules is their release behavior. The loaded capsules were incubated for 30 min either in 2 ml water or in 2 ml of a 3×10^{-2} M salt solution. The capsules were removed from the sample by centrifugation and the concentration of the PAH-Rh in both solutions was determined by fluorescence spectroscopy.

10 The dependence of the rhodamine emission on the salt concentration, was taken into account by means of adjusting the salt free solution afterwards to 3×10^{-2} M before the measurement were performed.

15 While in the pure water the amount of released PAH could not be detected almost all encapsulated PAH-Rh was released in 3×10^{-2} M NaCl. Confocal image proved that the interior of those capsules was void of fluorescent polymer again.

20 Example 4: Temperature dependent permeability

Monodisperse spheres ($d = 5.94 \mu\text{m}$) of a weakly polymerized melamine-formaldehyde resin were used as templates (Microparticle GmbH). The particles were covered by alternating adsorption of sodium polystyrene sulfonate PSS (MW 70 000 g mol⁻¹, Aldrich) and polyallylamine hydrochloride PAH (MW 70 000 g mol⁻¹, Aldrich) from an aqueous solution of 10^{-2} M PSS in 0.1 M sodium chloride. After 8 layers the core was dissolved in 0.1 M hydrochloric acid. The decomposition products were removed by washing with acid and an aqueous solution of 10^{-2} M NaCl.

25

30 Hollow polyelectrolyte capsules remained. In order to remove some defects in the capsules wall caused by mechanical stress during the dissolution two additional layers were coated on the capsules after the dissolution of

WO 02/17888

PCT/EP01/09908

- 33 -

the cores. Afterwards the capsules were washed extensively (8 times) with Milli-Q-water (resistance $>18 \text{ M}\Omega \text{ cm}^{-1}$). By this procedure, traces from salt and protons were essentially removed and a pH value of 6 was reached.

5 For the study of the permeability, fluorescence labeled polymers were used. PAH (MW 70 000, 1 rhodamine molecule per 245 PAH units and MW 15 000, 1 rhodamine per 320 PAH units) and PEI (MW 2 000, 1 fluoresceine molecule per 650 PEI units) were labeled by reaction with
10 rhodamine isothiocyanate or fluoresceine isothiocyanate. Labeled PSS (1 rhodamine molecule per 600 polymer units) was synthesized by copolymerization of styrene sulfonate with methacroyloxyethyl thiocarbamoyl rhodamine B (Polyscience). The MW of the polymer was determined being 120 000 g/mol by gel permeation chromatography.
15 Fluoresceine labeled human serum albumine (MW 69 000) and rhodamine labeled dextrane (MW 40 000 g/mol) were purchased from Aldrich company.

Laser scanning microscopy was performed for the determination of the
20 loading grade of the capsules. An inverse research microscope (Leica, Germany) with either a 40 x or a 100 x oil immersions objective was used. The amounts of polymer/monomer in the interior were determined by integration of the fluorescence intensity inside the capsules. The exact concentration was taken from calibration curves, determined for each
25 fluorescent probe separately. Due to the broad distribution of permeability for the capsules, statistical values were taken from 5 capsules each. Capsules, which could be recognized clearly as broken once (appr. 20%), were excluded from the determination. The temperature dependent measurements were done with an home-made aluminium device on top of
30 the sample, by which the temperature was settled by an electronic controller.

WO 02/17888

PCT/EP01/09908

- 34 -

Scanning force microscopy was applied to capsules, dried on air on a mica substrate. The drying process after the heat treatment at 60°C was done by transferring a drop of the capsule solution to the mica surface and drying without any change of the temperature.

5

4.1 Permeability

The permeability of the capsules was studied for positively (PAH) and negatively charged (PSS), zwitterionic (albumine) and noncharged (dextran) fluorescent polymers (dextran). In general, the capsules were mixed with the probe polymers in a concentration of about 2×10^{-3} mol/L with respect to the polymer units and incubated for 15 min. Then, the fluorescence intensity in the center of the capsules was measured by confocal imaging. The fluorescence events within the cross section of the capsule were integrated and compared with the fluorescence events in the bulk solution. Less fluorescence intensity inside than outside the capsules indicates an impermeability of the capsule wall for the probe polymer. Equal fluorescence intensities inside and outside of the capsules at the start of the experiment is caused by fast penetration of the polymers through the capsule wall, mostly observed for broken capsules. In case of dextrane as described below the fluorescence inside the capsules is higher than in the bulk phase indicating an accumulation of the polymer in the interior.

First the permeability of the capsule wall for the probe polymers was studied before any heat treatment. Within 24 hours the majority of capsules were impermeable for charged polyelectrolytes of MW above 60 000 g/mol independent on the sign of the charge. Figure 11a shows the confocal image for PAH of 70 000 g/mol. In the case of uncharged dextrane an unusual accumulation of the polymer inside of appr. 20% of the capsules was observed (Fig. 11b), while the other capsules exclude the polymer, too.

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WO 02/17888

PCT/EP01/09908

- 35 -

Further influence of the length of the polymer chain on penetration was determined. While within 1 hour 80% of the capsules are impermeable for PAH 70 000 g/mol, about 65% were impermeable for PAH 15 000 g/mol and for PEI 2000 all capsules were permeable. Small dye molecules such as fluoresceine, rhodamine and acridine orange can penetrate the capsule wall independent of their charge.

The dependence of the permeability of capsule walls on temperature was followed online by confocal imaging (in situ) by heating capsules in 10° steps from room temperature to 80°C. At each step the temperature was allowed to stabilize for 5 min. The changes in the polymer distribution within the capsules and the bulk solution were similar for each polymer and are demonstrated for heating the capsules in presence of rhodamine labeled PSS. Up to 50°C the polymers was excluded from the capsule wall, but at higher temperature the PSS starts to flow into the capsules until an equilibrium or even a slight excess was reached inside the capsules. The rate of penetration increases with the temperature furthermore. This is a clear proof, that the capsule wall can be opened for large polymer molecules very easy by increase of the temperature.

It was further examined whether the opening is an irreversible process or if the capsule walls can be closed again by reducing the temperature below 50°C. This was investigated by treating the capsules in absence of polymers at different temperature conditions deviating in time of heating and cooling.

Possible mechanisms leading to an increase of capsule wall permeability are discussed below. Besides the fact, that diffusion through a membrane increases generally with temperature due to the higher mobility of the polymer species, the rather strong effect observed in a narrow temperature range indicates structural changes in the polyelectrolyte wall. One possible mechanism could be the opening of pores in the polyelectrolyte membrane

WO 02/17888

PCT/EP01/09908

- 36 -

as it is described above for treatment with pH and salt for similar systems. It was observed that PSS/PAH capsules shrink at higher temperature by more than 10% in diameter. The mechanical stress at such process could cause also the formation of pores. In order to find such pores, the capsule wall was investigated by high resolution Scanning Force Microscopy after drying capsules on a mica substrate. Three samples were prepared before, during, and after heating to 60°C, but significant differences were not observed apart from the reduction of the capsules in diameter. Despite of this experimental result, the formation of pores can not be fully excluded because of the subsequent drying process can change the capsule structure remarkably.

Another mechanism is the weakening of the electrostatic bounds between polyanions and polycations. This leads to an increased charge density in the film, connected with stronger hydration, swelling and a better diffusion of the polymers through the wall. It was tried to support this mechanism by investigations of the resonance energy transfer in capsules consisting of 14 layers, in which the 10th layer is PAH-fluoresceine and the 12th layer is PAH-rhodamine. The spectra of the solution were taken before, during, and after heating to 60°C for 20 min. The spectra before and after the heating look quite similar, indicating an almost reversible change in the wall structure. The spectrum at 60°C deviates remarkably from the other spectra. The decrease of the rhodamine fluorescence indicates an increase of the averaged distance between fluoresceine and rhodamine molecules or the polymer layers, respectively. In order to exclude simple heating effects as origin of the observed spectra changes, a PAH polymer containing on every 106th position a fluoresceine molecule and on every 345th position a rhodamine molecule (MW 70 000 g/mol) was investigated under identical conditions.

The findings offer a unique possibility to encapsulate macromolecules, since the permeability of capsule walls was found to be controlled by

WO 02/17888

PCT/EP01/09908

- 37 -

temperature: the capsule wall is opened by heating and surrounding material can stream in. If the capsules are filled with the desired materials the wall can be closed again by cooling down the solution. Afterwards the remaining materials outside, e.g. polymers can be washed away and the

5 enclosed macromolecules remain inside the capsules. The encapsulation was done successfully for all labeled polymers by heating impermeable capsules (Fig. 12a) to 60°C for 20 min. After cooling and washing, the capsules contained the polymer as shown for example in Figure 12b. A

10 quantitative determination of the fluorescence intensity of the original polymer solution and the encapsulated polymer in the interior yielded a 30% of the bulk concentration in the interior. Both probe polymers used, i.e. labeled PSS as well as PAH were captured in the interior indicating a high reversibility of the permeability. Even storage of the filled capsules for

15 more than one month lead only to some loss of polymer. This loss was especially strong with more than 50% for PAH, but zero in case of dextrane.

WO 02/17888

PCT/EP01/09908

- 38 -

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WO 02/17888

PCT/EP01/09908

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WO 02/17888

PCT/EP01/09908

- 40 -

Table 1: Percentage of filled capsules after 24 hours incubation time in solutions of PAH-rho, 5×10^{-3} M and in presence of different salt concentrations.

Salt concentration / 10^{-4} mol/L	1	5	10	50	100	200
Percentage of filled capsules/ %	4.6	8.5	6	16.7	100	100

WO 02/17888

PCT/EP01/09908

- 41 -

Claims

1. A process for controlling the permeability of polyelectrolyte multilayer capsules, wherein at least one of the parameters of pH, temperature, light, salt concentration, ion composition, ion concentration, ionic strength, solvent composition or solvent concentration is adjusted or/and varied during preparation, storage or/and use of the capsules.
2. A process for controlling the permeability of polyelectrolyte multilayer capsules, wherein the number of polyelectrolyte layers or/and chemical composition, hydrophobicity, polarity, stiffness, molecular weight, the charge or charge density of layer constituents, in particular polyelectrolytes is adjusted in such a way that the desired permeability is achieved.
3. A process according to claim 1, wherein a pH of at least 8 is adjusted to render the polyelectrolyte multilayer capsules impermeable.
4. A process according to claim 1, wherein a pH of less than 6 is adjusted to render the polyelectrolyte multilayer capsules permeable.
5. A process according to any one of the preceding claims, wherein a salt concentration of at least 2×10^{-2} M is adjusted.
6. A process according to any one of claims 1-4, wherein a salt concentration of less than 5×10^{-3} M is adjusted.
7. A process according to any one of the preceding claims, wherein the temperature is set to at least 50°C.

WO 02/17888

PCT/EP01/09908

- 42 -

8. A process according to any one of claims 1-7, wherein the temperature is set to less than 50°C.
9. A process according to any one of the preceding claims, wherein a solvent composition containing an alcohol and/or water is used.
10. A process according to claim 2, wherein the number of polyelectrolyte layers is at least 8.
11. A process according to any one of the preceding claims, wherein lipids, surfactants, dyes, drug molecules, nanoparticles or/and biopolymers, such as polysaccharides, polypeptides, nucleic acids are included as layer constituents.

WO 02/17888

PCT/EP01/09908

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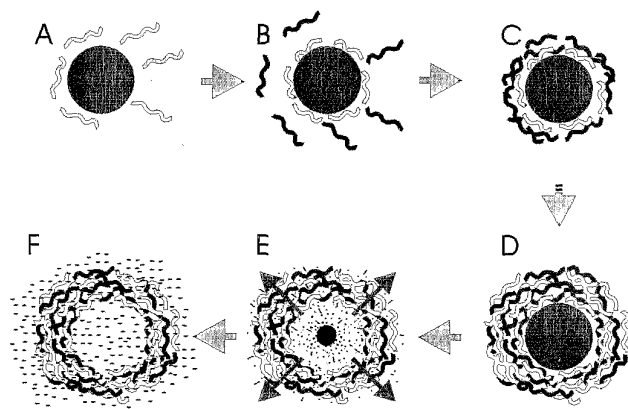


Fig. 1

WO 02/17888

PCT/EP01/09908

2/16

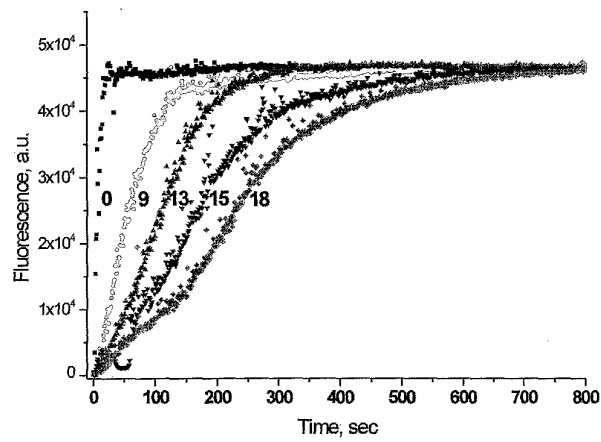


Fig. 2

WO 02/17888

PCT/EP01/09908

3/1 6

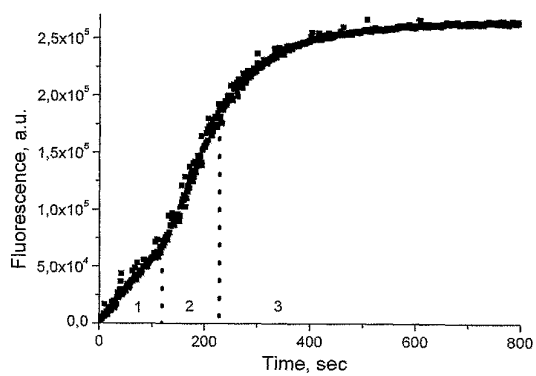


Fig. 3

WO 02/17888

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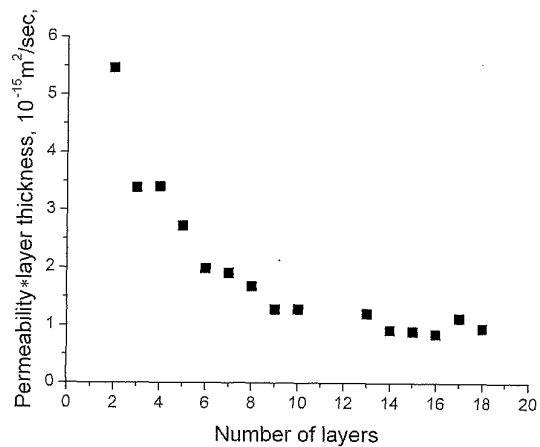


Fig. 4

WO 02/17888

PCT/EP01/09908

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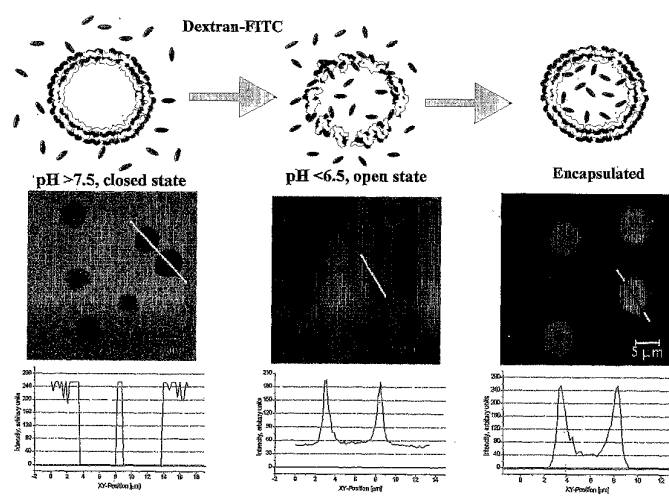


Fig. 5

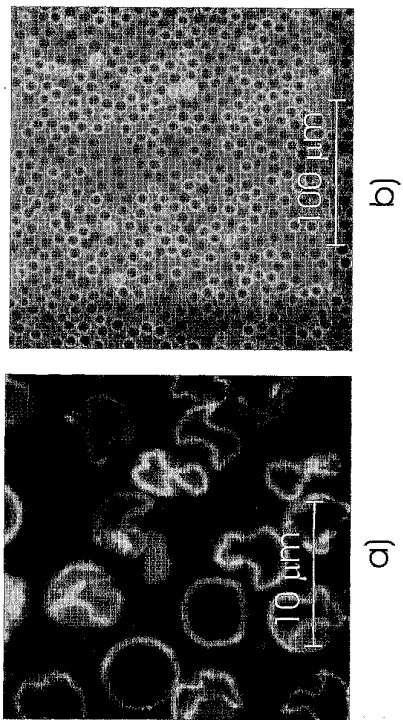
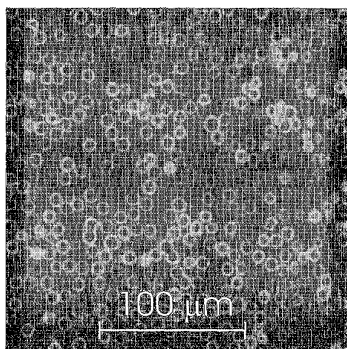


FIG. 6

WO 02/17888

7/16

PCT/EP01/09908



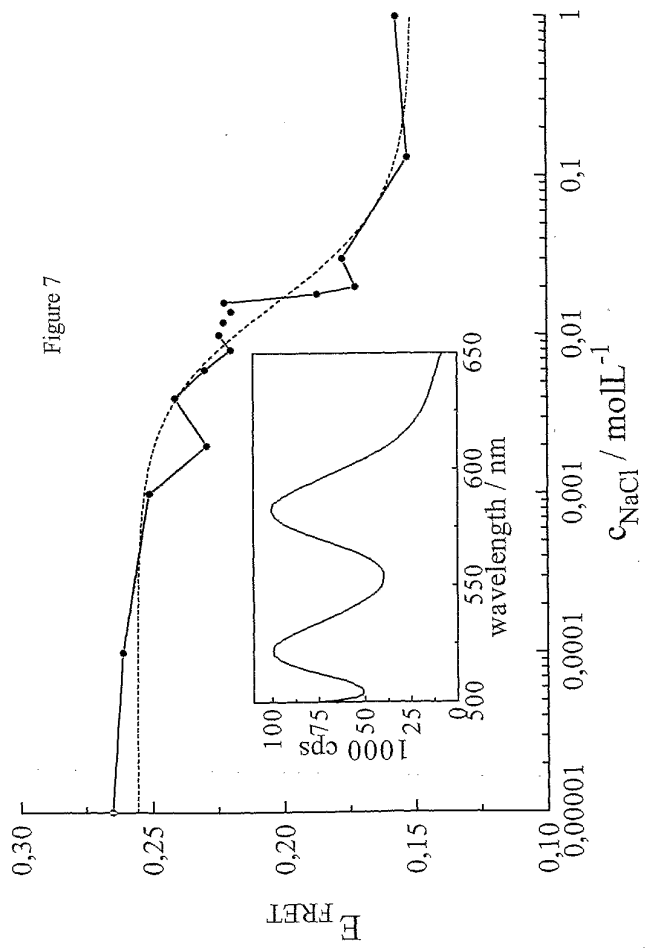
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FIG. 6

WO 02/17888

PCT/EP01/09908

8/16



WO 02/17888

PCT/EP01/09908

9/16

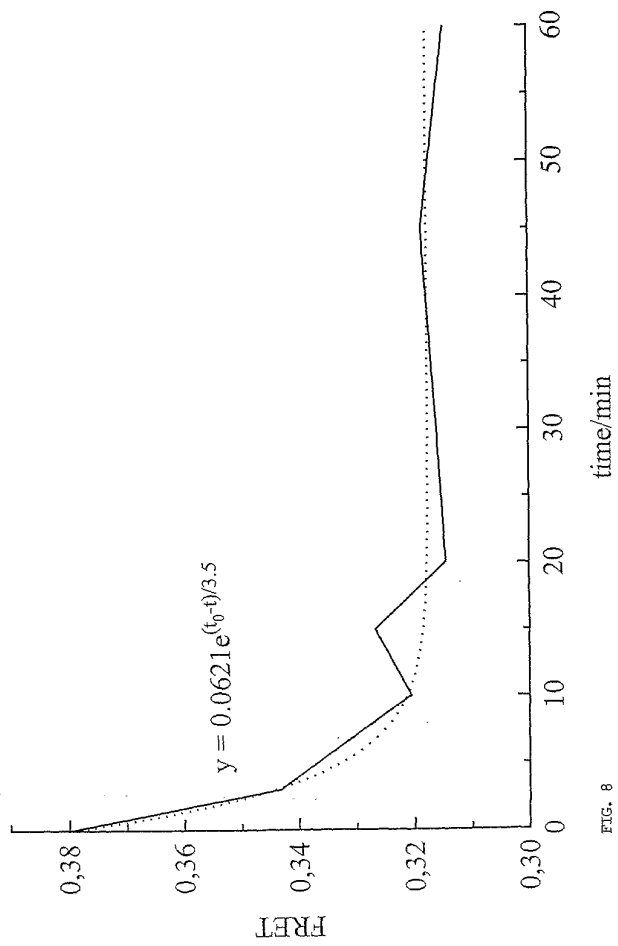


FIG. 8

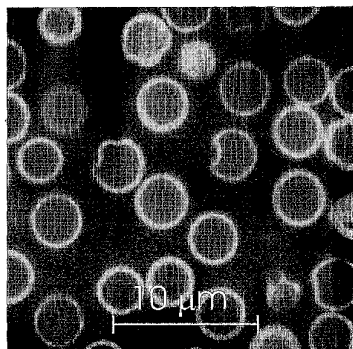


Figure 9

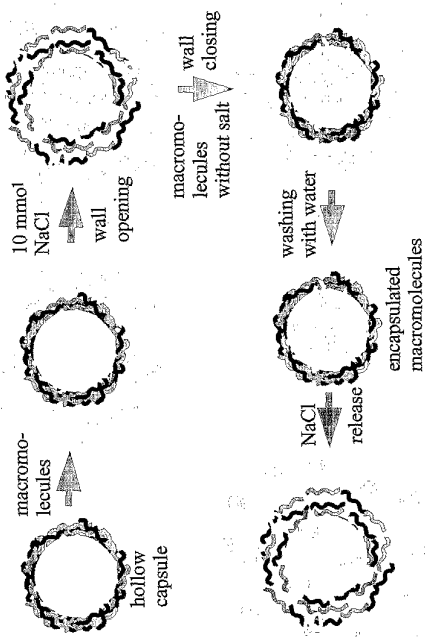


Fig. 10

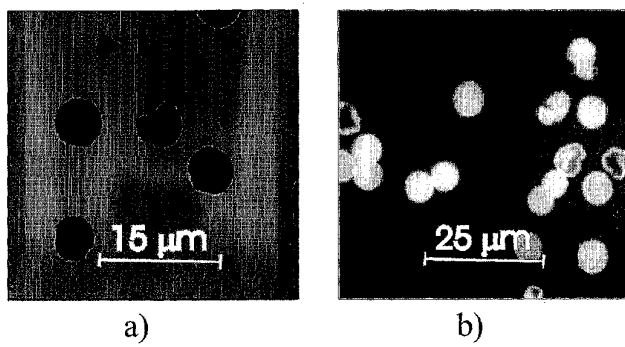


Figure 11

WO 02/17888

PCT/EP01/09908

13/16

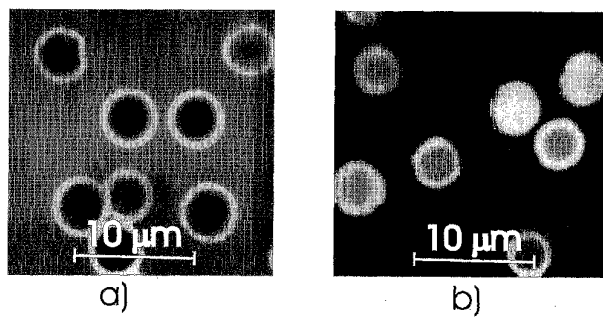


Fig. 12

WO 02/17888

PCT/EP01/09908

14/16

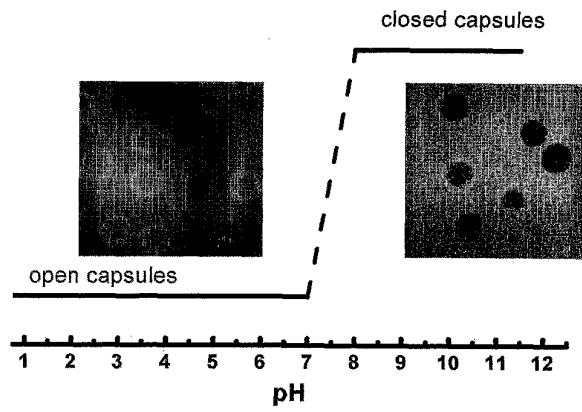


Fig. 13

WO 02/17888

PCT/EP01/09908

15/16

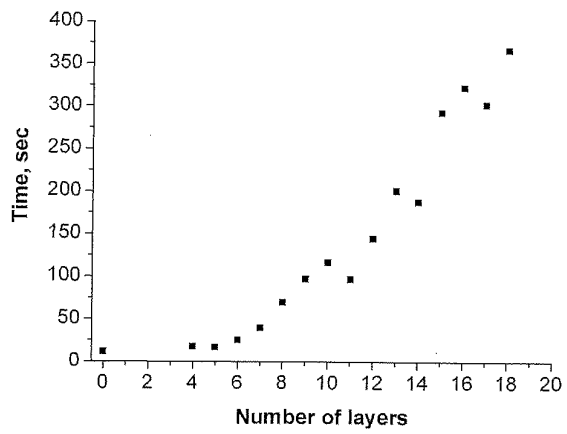
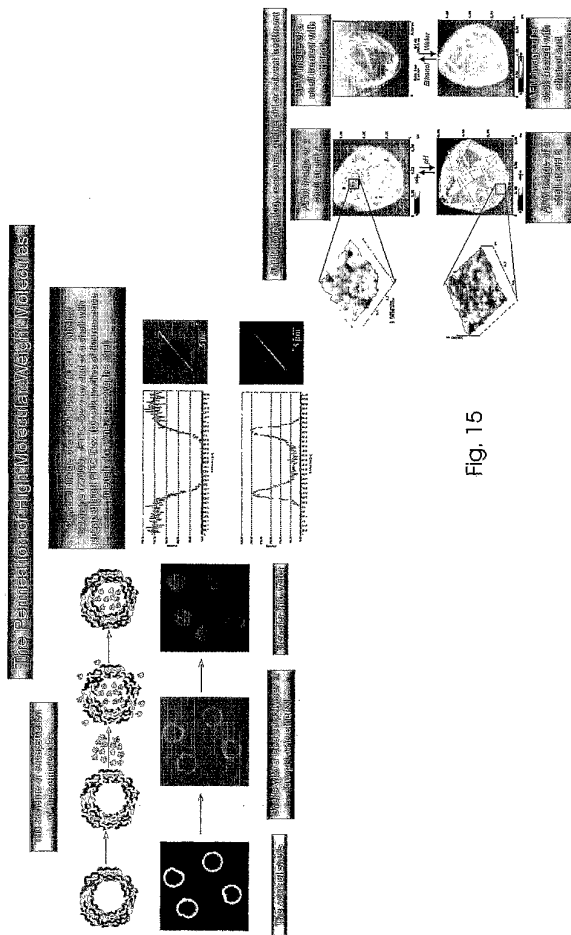


Fig. 14

WO 02/17888

PCT/EP01/09908

16/16



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Giesenhorst (DE). MÖHWALD, Helmuth [DE/DE];
Dr.-Gebauer-Strasse 21, 55411 Bingen (DE).

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(74) Agent: WEICKMANN & WEICKMANN; Postfach 860
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PLANCK-GESELLSCHAFT ZUR FÖRDERUNG
DER WISSENSCHAFTEN E.V. [DE/DE]; Hofgarten-
strasse 8, 80539 München (DE).

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(72) Inventors; and

(75) Inventors/Applicants (for US only): ANTIPOV, Alexei
[RU/DE]; In der Feldmark 7, 14476 Golm (DE). VIEIRA,
Euridice [PT/DE]; c/o Max-Planck-Institut für Kol-
loid-und Grenzflächen, enforchung, 14424 Potsdam (DE).
IBARZ, Gemma [ES/DE]; c/o Max-Planck-Institut für
Kolloid-und Grenzflächen, enforchung, 14424 Potsdam
(DE). SUKHORUKOV, Gleb [RU/DE]; Feuerbachstrasse
35, 14471 Potsdam (DE). DÄHNE, Lars [DE/DE];
Stillerzeile 3, 12587 Berlin (DE). GAO, Changyou(88) Date of publication of the international search report:
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(54) Title: CONTROLLED AND SUSTAINED RELEASE PROPERTIES OF POLYELECTROLYTE MULTILAYER CAPSULES

(57) Abstract: Method of layer-by-layer (LbL) assembly of oppositely charged polyelectrolytes was applied to coat fluorescein particles. These particles with a size of 4-9 µm were prepared by precipitation of fluorescein at pH 2. Polystyrenesulfonate (PSS) and polyallylamine (PAH) were used to compose the polyelectrolyte shell on the fluorescein core. The release of fluorescein molecules through the polyelectrolyte shell core dissolution was monitored at pH 8 by increasing fluorescence intensity. The number of polyelectrolyte layers sufficient to sustain fluorescence release was found to be 8-10. Sequentially adsorbed layers prolong core dissolution time for minutes. The permeability of polyelectrolyte multilayers of the thickness of 20 nm is about 10⁻⁸ m/s. Mechanism of fluorescence diffusion and osmotically supported release is under discussion. The features of release profile and possible applications of LbL method for shell formation in order to control release properties for entrapped materials are outlined. Also ambient conditions of pH, temperature and salt concentration were changed to control the permeability of polyelectrolyte multilayer capsules.

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5	Description
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A major task in the development of advanced drug formulations deals with the elaboration of delivering systems providing sustained release of bioactive materials. Mostly, these delivering systems comprise polymer particles in the size range of 10^2 to 10^5 nm. The drug molecules are embedded in polymer matrices or in core-shell structures. In the latter the shell degradation rate determines the release rate of the bioactive core material.

In principle, a shell around the active core can be fabricated by adsorption of polymers or biopolymers onto the drug particle surface [1] or by adsorption of the monomers with subsequent polymerization at the interface [2-4]. The composition of the shell may additionally provide certain functionalities. It may be adjusted to facilitate the interaction of the core with the solvent or to add certain desired chemical properties. The shell may also have magnetic, optical, conductive, or targeting properties for directing and manipulating the core containing bioactive material.

Recently, a novel type of shell structures constituting polymer capsules has
30 been introduced [5-7]. These novel hollow polymeric capsules have a
predetermined size in the sub-micron and micron range and tunable wall
properties [7,13-15]. These capsules are fabricated by means of layer-by-

WO 02/017888

PCT/EP01/09908

- 2 -

layer (LbL) assembling of polyelectrolytes onto colloidal particles with subsequent removal of the colloidal core. The layer-by-layer (LbL) assembling is performed by alternating adsorption of oppositely charged species, such as polyelectrolytes [16,1] onto the surface of colloidal particles. The driving force for LbL adsorption is the electrostatic attraction between the incoming polymer and the surface.

Various cores, e.g. organic or inorganic materials, biological cells, drug crystals or emulsion droplets, ranging in size from about 60 nm to tens of microns have been utilized as templates for multilayer formation by the LbL technique. Up to now a variety of different substances, such as synthetic and natural polyelectrolytes, biopolymers, proteins, nucleic acids, magnetic and fluorescent inorganic nanoparticles, lipids, etc. were employed as layer constituents to build the multilayer shell on colloidal particles. The thickness of the shell walls depends on the conditions of its preparation. It can be tuned in the nanometer range. The thickness of the multilayer films on colloidal particles can be adjusted in the nanometer range e.g. by adsorption of varying numbers of layers. It was established [8, 9] that the capsule walls have semipermeable properties. They are permeable for small molecules such as dyes and ions while they exclude compounds with a higher molecular weight [17,9].

There is a variety of materials, the encapsulation of which is desirable for application in different areas of technology, such as catalysis, cosmetics, medicine, biotechnology, nutrition and others.

One possible approach to load capsules with polymers is to embed the desired polymers within the inner layers of the shell structure while forming the capsules and to desorb polyelectrolytes, e.g. multivalent ions, from inner layers of empty shells into their interior [18]. Extraction of these ions results then in the release of the polymers into the capsule interior.

WO 02/017888

PCT/EP01/09908

- 3 -

Another approach is to directly synthesize polymers inside the capsules by taking advantage of the permselectivity of the capsule walls [19]. While capsules can be successfully loaded thereby these methods have only limited applicability with regard to the polymer species and often harsh
5 conditions have to be used for core dissolution or polymerization, being e.g. a low pH, oxidizing agents or organic solvents, which are used for core decomposition, or an elevated temperature during synthesis.

It would be desirable to provide systems having controllable or adjustable loading as well as release properties. In particular a method would be favourable which allows the loading or release of materials into and from capsules by modifying the capsule wall permeability. Further, for most applications a defined and controllable permeability of the capsule wall is required in order to control the process of loading the capsules as well as
15 any subsequent release under specific environmental conditions. Due to the fact that the loading is preferably fast, but the release should be in most applications slow, it is further desired that the permeability is switchable.

It was therefore an object of the present invention to provide methods to influence, vary or switch properties of capsule walls. Another object of the invention was to provide means to introduce macromolecules into the capsules and to switch and control capsule permeability for them.

These objects are solved by a process for controlling the permeability of polyelectrolyte multilayer capsules by variation of the reaction conditions during the preparation or use of the capsules, characterized in that at least
25 one of the reaction conditions of pH, temperature, salt concentration, ion composition, ion concentration, ionic strength, solvent composition or solvent concentration is varied.

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WO 02/017888

PCT/EP01/09908

- 4 -

The invention further relates to a process for controlling the permeability of polyelectrolyte multilayer capsules, wherein the number of polyelectrolyte layers is adjusted in such a way that the desired permeability is achieved.

5 According to the invention the permeability of polyelectrolyte multilayer capsules can be determined, varied or/and controlled by parameters of the environment of the capsules, e.g. reaction conditions during preparation or use of the capsules, media, in which the capsules are contained or into which the capsules are transported. It was found that the permeability to
10 high molecular weight compounds as well as low molecular weight compounds can be adjusted according to the needs in different applications.

As used herein high molecular weight compounds or macromolecules are
15 molecules having a molecular weight of at least 30 000 Da, more preferably at least 50 000 Da and most preferably at least 70 000 Da. Low molecular weight compounds or small molecules are molecules having a molecular weight of less than 10 000 Da, preferably less than 5 000 Da and more preferably less than 1 000 Da.

20 The permeability control according to the invention offers a unique tool for entrapping molecules within capsules and releasing them in a predetermined manner, e.g. over an extended period of time or at a desired, predetermined site or time point.

25 The process according to the invention enables particularly a reversible amendment of the permeability of capsules. This enables specifically charging the capsules with desired active substances or specifically releasing active substances entrapped in the capsules, respectively, by
30 amending the environmental conditions of the capsules in a simple way. Thus, e.g. a permeability increase makes it possible to later charge the finished capsules with active substances. Such an "open condition",

WO 02/017888

PCT/EP01/09908

- 5 -

wherein active substances can permeate through the capsule walls is present according to the invention when over 50%, more preferably over 70% and most preferably over 90% of the capsules are permeable. After the capsules have been charged, e.g. during storage or transport, the permeability of the capsule wall to the entrapped active substance can be reduced by adjusting of suitable conditions so that no active substance can leave the capsules. Such a "closed condition" of the capsules, however, at the same time prevents that further, possibly undesired substances can enter the capsules. At the desired time and site of release, respectively, the active agent can be released in a defined way, e.g. delayed, by increasing the permeability of the capsule walls. Further, it is possible to obtain a release in certain compartments of cells or certain areas of an organism by using capsule walls exhibiting high permeability to the entrapped active substance under conditions prevalent in the desired tissue.

According to the invention the permeability of capsule wall and thereby the incorporation or exclusion of macromolecules can be tuned by environmental conditions in a defined manner. Capsules composed of polyelectrolytes, the charge of which depends on the pH, can be used for a pH-controlled uptake and release of macromolecules.

The practical use of polyelectrolyte capsules for sustained drug release requires quantitative data on the permeation of small molecules through polyelectrolyte walls, which are presented herein.

In the light of sustained release it would be advantageous to be able to decrease the layer permeability for small polar molecules once they are encapsulated. One possible way to approach this goal is the use of lipids as a layer constituent [9, 10]. Herein, the formation of thicker capsule walls is presented in detail being a favourable and easy way to decrease permeation. It was found that increasing the layer number will decrease penetration of the shells by encapsulated material or molecules.

WO 02/017888

PCT/EP01/09808

- 6 -

To verify this approach fluorescein microparticles were covered with a different number of polyelectrolyte layers. Dyes like fluorescein can be considered as model substances for a large class of drugs. Afterwards core dissolution was initiated by a pH change and monitored by the increasing
5 fluorescence in the bulk.

It was found that polyelectrolyte multilayer shells assembled around cores consisting of low molecular weight compounds provide barrier properties for release under conditions where the core is dissolved. This finding is a
10 novel approach for fabrication of systems with prolonged and controlled release properties. The release can be adjusted with the number of assembled polyelectrolyte layers. The capsule permeability for low molecular weight compounds was found to depend strongly on the number of polyelectrolyte layers in the capsule.

15 A large variety of synthetic polyelectrolytes with different properties, lipids, and polysaccharides have been already used for multilayer assembly [9, 12]. This provides many possibilities to tune the release properties of the shells together with ensuring biocompatibility and possibility of using
20 various cores. The assembling of shells by LbL technique opens new pathways for biotechnological applications, where controlled and sustained release of a substance is required. Many problems connected with drug formulation, release, and delivery, controlling the concentration in the organism and periodicity of its reception can be solved by the formation of
25 shells on precipitates and nanocrystals. Further it is not difficult to add to the polyelectrolyte layer targeting properties. This way the affinity of polyelectrolyte multilayer coated drugs to specific or injured tissues can be increased.

30 In the following the release properties of capsules with regard to the number of layers forming the capsule wall are discussed in more quantitative terms using fluorescein as model substance (MW ~ 350 Da).

WO 02/017888

PCT/EP01/09908

- 7 -

The permeation of a molecule such as fluorescein through the shell wall is described by its permeability (P). Equation (1) combines a flux (J) with parameters of the system and the rate of change of fluorescein concentration, c^i , in the bulk. When this rate is constant one may easily calculate the permeability (P) from the slope of the fluorescence increase (part 2 of Fig.3).

$$\frac{dc^i}{dt} V_0 = J = P \cdot (c^i - c^o) \cdot S \quad (1)$$

where V_0 is the volume of solution, J is the fluorescein flux through the capsule walls with the total surface area of S, and $(c^i - c^o)$ is the difference of the fluorescein concentration inside (c^i) and outside (c^o) the capsules.

As long as a core of solid fluorescein is present within the capsules, the interior of the capsules contains a saturated fluorescein solution, c^s , of 25 mg/ml. Hence, the concentration difference at the beginning of the process in the right side of Eq.1 can be safely replaced by $c^i = c^s$. The capsules were assumed spherical with an average diameter 5 μm . The permeability can thus be calculated from

$$P = \frac{dc^i/dt \cdot V_0}{c^s \cdot S} \quad (2)$$

For 8 to 18 layers the permeability value was in the order of 10^{-8} m/s. Assuming single polyelectrolyte layer thickness of 2 nm the permeability can be converted into a diffusion coefficient (D) by means of multiplying the permeability with the shell wall thickness. The calculated diffusion coefficients are in the order of 10^{-15} m²/s.

If the permeability of the polyelectrolyte multilayer is provided by diffusion through the entangled polymer network, it should scale with the inverse of the layer thickness. The behavior of the permeability times thickness as a function of the number of layers is shown in Figure 4. As can be seen, the

WO 02/017888

PCT/EP01/09908

- 8 -

permeability decreases with increasing layer number much faster than expected from a straightforward thickness increase. Only from approximately 8 layers onwards the permeability multiplied by the shell thickness becomes a constant indicating that the permeability is now controlled by the thickness increase, for example, the diffusion limiting region is the polyelectrolyte layer. This finding is consistent with the earlier observations [11] where it was shown that the conformation of the first eight layers differs from that of further assembled layers. These deeper layers are more dense resulting in a fivefold reduction of the estimated diffusion coefficient as can be inferred in Fig. 4.

A permeability coefficient might also be calculated from the release profile at the third stage of the fluorescein release curve. It can be expected that the time dependence of the release at this stage is exponential:

$$c^e(t) \sim \left(1 - e^{-\frac{\bar{S}}{V} P t} \right), \quad (3)$$

where $\bar{S}$ and V represent the surface and the volume of an average capsule. However, the permeability estimated from this equation was one order less than the one calculated from the slope of the linear region. There are currently two possible explanations for this discrepancy. Either the polydispersity of the particles contributes to an apparent prolonged release, because the characteristic time release $V/\bar{S}P$ increases linearly with the particle radius. Or, the relaxation of the osmotic stress towards the end of release may reduce the permeability.

In the following the permeation mechanism is discussed in more detail. One may distinguish between diffusion through water filled pores and a "bulk" diffusion mechanism through homogeneous phase of polyelectrolyte multilayer shell. For thinner walls the drastic dependence on shell thickness

WO 02/017888

PCT/EP01/09908

- 9 -

may be explained either by existence of pores that are successively closed by further layer deposition or by thickness dependent diffusion coefficient. The latter was indeed observed with permeation studied by planar polyelectrolyte films where typical values of D between 10^{-19} and 10^{-20} m²/sec were derived [11]. In order to compare our results with those of the previous study we should remark the following differences.

- According to the method described in [11] the films were prepared by drying after each adsorption step which is not possible during particle coating and which leads to a denser film.

- The measurement consisted in depositing a dye probe in a defined depth into the film and observing time dependent fluorescence changes due to quencher diffusion. For sufficiently small pore concentration, which is surely the case here, this technique is only sensitive to "bulk" diffusion and will not reflect the permeation through pores.

Thus one may expect that the diffusion coefficients derived from permeability data are larger than values measured in [11]. Still, this cannot explain the difference of 3-4 orders of magnitude and therefore they more probably correspond to diffusion through pores. Comparing the diffusion coefficient D with that in bulk water ($\sim 10^{-10}$ m²/s) which is an upper limit for the diffusion in the pore volume we may estimate the fraction of pore volume inside the walls larger than 10^{-5} .

Next a possible mechanism of pore formation is addressed. A destabilization of the wall may be expected, since on increasing the pH towards 8 the aminogroups of PAH may deprotonate and this may result in pore formation within the multilayers. Another possibility of pore formation is the osmotic pressure due to the water coming to the interior as a result of fluorescein core dissolution. The related hydrostatic pressure difference creates a tension in the wall which may widen existing or create new

WO 02/017888

PCT/EP01/09908

- 10 -

pores. As seen on Fig.3 (part 1), especially for bigger numbers of layers, in the beginning of dissolution the release is sustained. It is assumed that the polyelectrolyte shell suffering osmotic pressure from inside resists release of fluorescein until the fluorescein molecules develop a path out of capsules. The pores are formed as a result of this rearrangement of polyelectrolyte multilayers. It should be noted that templates and the resulting capsules have different diameters and also heterogeneous wall thicknesses. The diffusion coefficient is thus an average but there may still be larger templates with slower release profile.

In summary, it can be noted that by increasing the number of layers of polyelectrolyte capsule walls the permeability of the capsules can be reduced. For a delayed release preferably capsules having ≥ 8 , more preferably ≥ 9 and most preferably ≥ 10 layers are used.

In a preferred embodiment the invention relates to a novel approach for encapsulating materials and molecules, such as macromolecules, biopolymers, drugs etc., in pre-formed hollow polyelectrolyte capsules. According to this embodiment of the invention the material or/and molecules can be introduced in hollow capsules rather than forming capsules around the materials/molecules.

The properties and structure of polyelectrolyte multilayers are found to be sensitive to a variety of physical and chemical conditions of the surrounding media. In particular, the pH is one of the physico-chemical parameters, which influences the state of the inter-polyelectrolyte complex [20,21], especially in the case, if the charge of one polyelectrolyte in the complex depends on the pH. The polyelectrolyte pair, poly(styrene sulfonate) (PSS) and the relatively weak polycation poly(allylamine hydrochloride) (PAH), has been most extensively used for producing multilayer films on flat and colloidal surfaces (1) and serves as exemplary

WO 02/017888

PCT/EP01/09908

- 11 -

polyelectrolyte pair for illustrating the invention. However, the invention is not limited to this specific polyelectrolyte pair.

By using different polyelectrolytes, the permeability can be further modified. The combination of cationic and anionic polymers results in almost unlimited variation possibilities. Characteristic values, such as polarity and polymeric rigidity can be adjusted via the chemical composition and have a specific effect on different substance classes. For example a reduction of the polarity results in a higher hydrophobicity, which decreases the permeability for polar, water soluble substances, whereas the permeability for unpolar, oil soluble substances is enhanced.

In addition, the permeability behaviour of the capsules can be strongly modified via the molecular weight and the degree of branching of the polyelectrolytes used. The molecular weight is advantageously set between 10 000 and 500 000 g/mol, wherein generally higher molecular weights and increasing branching lead to higher capsule stability and lower permeability.

By varying the pH of the medium, the permeability of the walls of microcapsules can be varied. While it is not intended to be bound to a specific mechanism of the pH-induced permeability change, it is supposed that changes of the polyelectrolyte charge upon pH variation are able to induce pore formation (22) or loosen the polyelectrolyte network, thus enabling polymer penetration. An influence of the ion concentration going along with the pH change may also contribute to the permeability change.

This possibility of switching the capsule walls between an open and closed state provides a convenient and efficient tool to control the uptake and release of molecules, in particular polymers, biopolymers and nanoparticles. For instance, the capsules might be loaded at low pH and after increasing the pH the material is captured inside. For release the pH can be slightly

WO 02/017888

PCT/EP01/09988

- 12 -

decreased again, whereby the kinetics of release can be tuned by the pH. The herein demonstrated possibility of controlling loading and release of macromolecules into and from polyelectrolyte capsules allows for widespread application. The described pH-induced permeability change of the polyelectrolyte network in the film is a general mechanism for
5 modifying the permeability of polyelectrolyte multilayers and is not limited to a specific composition of the shell. Investigations on flat polyelectrolyte films made from weak polyelectrolytes have shown that pores can be created by changing the pH or the salt concentration.

10 We have found that permeability of capsules composed of polyelectrolytes, in particular of weak polyelectrolytes, strongly depends on the pH. Being impermeable for macromolecules at a pH when the charges of two polyelectrolytes forming the capsule wall are compensated, capsules are
15 open at a pH, at which a weak polyelectrolyte is completely charged. This dependence gives an opportunity to encapsulate polymers by opening and closing a capsule by means of pH. By means of AFM at the open state pores ranging in size from 100 to 300 nm were observed in the capsule wall. A further remarkable feature of the process of pore formation by pH
20 variations is that the pore formation or increased permeability is reversible.

Further permeability of the capsules inversely depends on the number of layers they consist of. Employment of both these factors gives a possibility to regulate capsule release properties for low as well as for high molecular
25 weight compounds. Fluorescein particles, stable at acidic pH can be used as core. They can be covered with different numbers of polyelectrolyte layers. The kinetics of fluorescein dissolving and release was measured at base pH. It was found that time of core decomposition depends strongly on the capsule wall thickness. During the dissolving a sustained release of
30 fluorescein was observed.

WO 02/017888

PCT/EP01/09908

- 13 -

Permeability control by means of pH and capsule wall thickness is proposed as a new approach for controlled uptake and release of different substances into and from capsules. It opens avenues for using polyelectrolyte capsules for many applications, such as drug carriers, microreactors and so on.

In summary, it can be stated that by increasing the pH a reduced capsule permeability can be achieved and by reducing the pH an increased capsule permeability can be achieved, whereby this process is reversible. It was found that capsule walls particularly at a pH of > 8 , more preferably at a pH of > 10 are impermeable (closed condition), whereas at a pH of < 6 , particularly of < 4 a permeability (open condition) of the capsule walls can be achieved. The change in pH represents a simple way for adjusting the permeability and by amending the surrounding medium makes it possible to provide for a permeability switch for the capsules.

This application also presents a novel, simple and very gentle method for targeted encapsulation and release of sensitive, macromolecules by controlling the capsule wall permeability by the salt concentration. Salts have an immense importance in the living organisms. The salt concentration in the cytoplasm and in the extra cellular medium is strongly regulated by the permeability of the cell membranes, by osmosis and by active transport via energy driven pumps. The physiological concentration of sodium chloride in plasma is about 0.15 M, other ions, such as potassium or calcium are crucial for signal transduction across the membrane and inside the cell.

The permeability of hollow polyelectrolyte capsules, preferably of capsules made from PAH/PSS can be reversibly switched between an open and closed state by varying the salt concentration in a narrow concentration range between 0.5 ... 2×10^{-2} M/l. The increase of the salt concentration

WO 02/017888

PCT/EP01/09908

- 14 -

leads to a reversible opening of the capsule wall for large molecules of MW above 50 000 Da, preferably above 70 000 Da.

5 The absence of pores in scanning force microscopy images, the relatively small permeability and the step-like decrease of the Förster resonance energy transfer in the wall with increasing salt concentration indicates a weakening of the electrostatic interactions between the polyelectrolytes and a subsequent swelling connected with a much better diffusion of the polymers. Salt triggered permeability is a very easy, important, and
10 effective way for the encapsulation and release of, especially, sensitive macromolecules such as enzymes, proteins, or DNA in micro- and nanocontainers.

In summary, it can be noted that by increasing the salt concentration an
15 increase of the permeability of the capsule walls can be achieved, whereas reducing the salt concentration causes a reduction of the permeability (closed condition). Preferably the capsule walls are made permeable by a salt concentration of at least 2×10^{-2} M, more preferably of at least 3×10^{-2} M and most preferably of at least 1×10^{-1} M. If a closed condition of
20 the capsules is desired, the surrounding conditions are adjusted to a salt concentration of at most 5×10^{-3} M, more preferably of at most 1×10^{-3} M and most preferably of at most 1×10^{-4} M.

Suitable salts according to the invention are all heteropolar compounds
25 comprising at least one anion and at least one cation. The anions and cations can be singly charged or multiply charged ions. For adjusting the permeability preferably inorganic salts are used, particularly metal salts, such as metal halogenides, particularly alkali metal halogenides. Examples of particularly preferred salts are salts containing an alkali metal cation,
30 particularly Li^+ , Na^+ , K^+ , an earth alkali metal cation, particularly Mg^{2+} , Ca^{2+} or a different metal cation, e.g. Al^{3+} , an iron ion, etc. and an anion, e.g. a halogenide anion, or a different anorganic anion. The metals may

WO 02/017888

PCT/EP01/09908

- 15 -

also be replaced by other positively charged groups, e.g. an ammonium group, a sulfonium group or a phosphonium group. However, it is also possible to use organic salts, particularly salts containing organic anions.

5 Apart from the salt concentration, the permeability can also be adjusted by the ion concentration and ion composition of the environment, respectively, wherein also here it was detected that for higher ion concentrations a permeability is achieved, whereas for low ion concentrations an impermeable capsule wall was obtained.

10 The ion composition particularly plays a role, since the permeability of the capsules is influenced by interactions between ions of the surrounding medium and the charges of the polyelectrolyte shells. By suitably selecting the ions of the surrounding medium, e.g. ions forming complexes with the polyelectrolytes of the capsule shells, the permeability can be adjusted,

15 The dependence of permeability on salt concentration, ion composition and ion concentration can be particularly used advantageously by administering encapsulated active substances to organisms, e.g. mammals and particularly humans. Therefor capsules can first be charged with active substances, wherein in vitro the capsule walls are made permeable to the active substance with the help of the above described measures. Then the encapsulated active substances can be stored in a stabil form, e.g. by adjusting a salt concentration of the storage medium to $< 5 \times 10^{-3}$ M. This can be done e.g. by storage in distilled, preferably in sterile distilled water. 20 The administration of charged capsules is accompanied by an amendment of the surrounding medium, wherein, as already stated above, the physiological concentration of sodium chloride in plasma is about 0.15 M. Under these surrounding conditions the capsules are permeable to the active substance so that the active substance is released in the target 30 tissue.

WO 02/017888

PCT/EP01/09908

- 16 -

The invention also relates to a simple method for the polyelectrolyte capsules allowing opening and closing of the wall for large polymers but also for small organic molecules and to control the permeability in a simple way by temperature treatment.

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It has been shown, that the permeability of thin polyelectrolyte walls can be switched easily by heat. This is probably caused by an temporarily swelling of the wall due to stronger dissociation of the cation-anion bonds at higher temperature, followed by hydration of the formed charges and entanglement of the polymer chains. The reversibility of this process provides an excellent tool for loading hollow polyelectrolyte capsules with large macromolecules, polymers or nanoparticles for drug encapsulation as well as for the controlled and intelligent release of active substances. For example, in the agriculture many pests are active mainly at high temperatures. Encapsulation of pesticides in such capsules can ensure a release only at hot and sunny days. Or, in the new generation of intelligent clothes, deodorants will be released only in case of strong sweating.

Preferably at a temperature of up to 50°C, more preferably up to 40°C, the permeability is low (closed condition), whereas at a temperature of above 50°C, particularly of at least 60°C, permeable capsules (open condition) are obtained. By increasing the temperature in a simple way a permeability increase can be achieved.

In a further embodiment of the invention the permeability of polyelectrolyte multilayer capsules is amended by varying the solvent composition or solvent concentration. Thus, the release behaviour can be adjusted via the solution, wherein as solution e.g. polar solutions, particularly water or/and polar solutions or mixtures thereof can be used. Particularly suitable solutions for adjusting the release behaviour are alcohol/water mixtures, wherein the alcohol content, e.g. methanol, ethanol or propanol, can be adapted to the individual application.

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WO 02/017888

PCT/EP01/09908

- 17 -

Further, the permeability of polyelectrolyte multilayer capsules can further be influenced by adding surfactants, such as sodium dodecylsulfate (SDS) or dipalmitoyl-DL- α -phosphatidyl choline (DPPC) to the surrounding medium or as coating for the core used in capsule production.

5 The permeability of polyelectrolyte multilayer capsules can also be influenced by light. Photosensitive groups in the electrolyte layer, such as azo compounds or organic bisazides can be modified chemically by light, which may lead e.g. to an increase of the permeability or to a reduction of
10 the permeability, if the photosensitive compositions are selected which cross-link by exposure to light.

As explained above, according to the invention it is possible to insert further components into the polyelectrolyte layers in order to obtain a
15 broad modification of the permeability. The further components can e.g. be bound covalently to the polyelectrolyte or be independent molecules or particles, which are only embedded in the polyelectrolyte layers. The further components can be incorporated together with the polyelectrolyte or as independent units by using various methods, preferably the layer-by-
20 layer method, but also a single step method or subsequent diffusion.

The microcapsules, in particular hollow microcapsules, whose permeability
25 can be controlled according to the invention preferably can be fabricated by alternating deposition of oppositely charged polyelectrolytes on soluble colloidal templates. The polyelectrolyte capsules can e.g. be prepared by covering templates, ranging e.g. from 60 nm, preferably from 100 nm, to 10 μ m, preferably to 2 μ m in size, with alternating layers of polycations and polyanions [6,7]. After formation of a polyelectrolyte wall of sufficient thickness, the templates can be dissolved and hollow capsules are
30 obtained. The templates can e.g. be removed by changing the pH or by oxidative decomposition of the template. This is possible due to the remarkable property of the capsule wall to be permeable for small

WO 02/017888

PCT/EP01/09908

- 18 -

molecules having a molecular weight of less than 10 000, preferably less than 5 000 and particularly less than 1 000 but not for polymers. The template determines size and shape of the capsule. Monodisperse capsules in the range from 500 nm to 10 μm can be prepared. Suitable capsules and their production and composition are described, e.g. in WO99/47252, WO99/47253, WO00/03797 and WO00/77281, the disclosure of which is herein incorporated by reference.

A remarkable feature of the capsule wall is that their properties can be tuned within wide ranges by varying the layer material and/or number or/and by varying environmental conditions such as pH, salt concentration, ion strength, ion composition, solvent concentration, solvent composition or/and temperature. These capsules offer broad perspectives in nanoscale encapsulation of drugs, enzymes, DNA, minerals, dyes, polymers, proteins and/or other active macromolecules.

The capsules are preferably fabricated by alternate adsorption of oppositely charged polyelectrolytes onto the surface of colloidal particles. Different cores having a size varying from 0.06 preferably from 0.1 to 10 μm , such as inorganic colloidal particles, biological cells, protein aggregates, drug nanocrystals can be used. Hollow capsules can be produced by subsequently dissolving the core.

The permeability of capsules is of essential interest because of its significant importance in diverse areas, relating e.g. to biotechnology, medicine, food industry, etc.

The invention shall be further illustrated by the following examples and figures:

Fig. 1: Scheme of the polyelectrolyte multilayer deposition process and of the subsequent core dissolution. The initial steps (A-D) involve stepwise

WO 02/017888

PCT/EP01/09908

- 19 -

shell formation on a fluorescein core. After the desired number of polyelectrolyte layers is deposited the coated particles are exposed to pH 8 (E) and core dissolution with fluorescein penetration into the bulk is initiated resulting finally in fully dissolved cores and remaining empty capsules (F).

Fig. 2: Fluorescence increase upon time, obtained by dissolving fluorescein particles covered with shells of different thickness (9, 13, 15, and 18 layers).

Fig. 3: Three stages of fluorescein core dissolution covered with 17 PSS/PAH layers.

Fig. 4: Fluorescein diffusion as a function of layer number.

Fig. 5: Permeation and encapsulation of FITC-dextran (M.w. 75 000) into polyelectrolyte multilayer capsules. Left - pH = 10, center - pH = 3, right - pH increased to 10 after the capsules were loaded with FITC-dextran at pH = 3. The bulk FITC-dextran was removed by washings at pH = 10. Top - scheme, center - confocal images of the capsules, bottom - fluorescence profile along the line indicated in the confocal images.

Fig. 6: Confocal Laser Scanning Microscopy images showing: a) capsules for which the washings and the core dissolution was performed in the presence of 0.05 M NaCl; b) capsules which were washed with pure water after each deposition step; 24 h incubation in a 10^{-2} M PAH-Rho solution; c) same procedure as in b) but incubation in presence of 10^{-2} M salt.

Fig. 7: Efficiency of the Förster Resonance Energy Transfer in capsules containing the 10th layer PAH-fluo and the 12th layer PAH-rho in dependence on the salt concentration. The inset shows a typical fluorescence spectrum of these capsules. Excitation was set at 495 nm.

WO 02/017888

PCT/EP01/09908

- 20 -

Fig. 8: Time-dependent decrease of the FRET efficiency as a function of the salt concentration.

Fig. 9a): Capsules, loaded with 5×10^{-3} M PAH-rho according to the protocol shown in Fig. 9b) Same capsules after release of the encapsulated PAH-rho induced by a 3×10^{-2} M salt solution.

Fig. 10: Scheme of encapsulation and release of macromolecules by switching the permeability salt concentrations changes.

Fig. 11a): CLSM image of capsules in presence of 10^{-3} M Fluorescein-PAH (MW 70 000 g/mol, polymer exclusion); b) CLSM image of capsules in presence of 10^{-3} M Rhodamine-Dextran (MW 50 000 g/mol, accumulation);

Fig. 12a): CLSM image of capsules in presence of 10^{-3} M Rhodamin-PAH (MW 70 000 g/mol) at 23°C; b) encapsulated Rhodamine-PAH in capsules after heating the solution for 20 min to 60°C and subsequent washing with water.

Fig. 13: Permeability of capsules composed of 8 layers of PSS-PAH for a labeled dextran, molecular weight 77 000 at a low and high pH.

Fig. 14: Characteristic time of dissolving of fluorescein core, covered with different number of PSS-PAH polyelectrolyte layers.

Fig. 15: The permeation of high molecular weight molecules.

Table 1: Percentage of filled capsules in solutions of PAH-rho, 5×10^{-3} M, after 24 hours incubation time and in presence of different salt concentrations.

WO 02/017888

PCT/EP01/09908

- 21 -

Examples

Example 1: Varying number of polyelectrolyte layers

5 Materials

Polyelectrolytes. Sodium poly(styrene sulfonate) (Na-PSS, MW ~70 000), poly(allylamine hydrochloride) (PAH, MW ~50 000), and fluorescein, sodium salt, were obtained from Aldrich. Ethanol, sodium chloride, boric and hydrochloric acid were purchased from Sigma. All materials were used without further purification.

The water used in all experiments was prepared in a three stage Millipore Milli-Q Plus 185 purification system and had a resistivity higher than 18.2 MΩ cm.

15 Methods

Fluorescein particles were prepared by addition of one part of ethanol and four parts of hydrochloric acid, pH 2, to one part of 15 mg/ml aqueous fluorescein solution. The particle size was measured by optical microscopy.

20 The 4-9 μm in diameter fluorescein particles were washed in hydrochloric acid by 5 repeated centrifugation cycles at 450 g. To prevent preliminary core decomposition all further multilayer deposition and washings were performed in hydrochloric acid at pH 2.

25 Polyelectrolyte multilayer assembly. The multilayer assembly was accomplished by adsorption of polyelectrolytes at a monomer concentration of 10^{-2} M in 0.5 M NaCl, pH 2. Oppositely charged polyelectrolyte species were subsequently added to the suspension of fluorescein particles followed by repeated centrifugation cycles in
30 hydrochloric acid [7]. Fluorescein particles were allowed to interact with polyelectrolyte solution for 15 minutes. Fluorescein particles were

WO 02/017888

PCT/EP01/09908

- 22 -

centrifuged at 700 g for 10 minutes. Gentle shaking followed by 1 minute ultrasonication was used to disperse particles after centrifugation.

5 Fluorescence spectroscopy. The core dissolving was conducted in H_3BO_3 -NaCl-NaOH buffer, pH 8. The kinetics was followed by recording the time dependence of the emission at 522 nm. Excitation was set at 488 nm.

10 Confocal microscopy. Confocal images of capsules after dissolving the core were obtained by means of a Leica confocal scanning system. A 100 x oil immersion objective with a numerical aperture of 1.4 was used.

Figure 1 provides the scheme of fluorescein particles encapsulation and release. After LbL adsorption (Fig. 1A-1D) core dissolution is initiated by 15 changing the pH from pH 2 to pH 8 (Fig. 1E) and completed after a certain period of time (Fig. 1F).

Fluorescein particles rapidly dissolve at pH 8. The idea was thus to slow down the rate of core dissolving by covering the particles with a 20 polyelectrolyte multilayer. Shells walls consisting of a different number of layers were fabricated and examined with regard to their fluorescein permeability behavior. Fluorescence spectroscopy is a convenient tool for the determination of the core dissolving rate because the fluorescence of the core is completely suppressed as a consequence of the self-quenching 25 of the dye. Upon releasing the dye into the bulk the fluorescence intensity increases. Thus the rate of cores dissolving can be directly followed by measuring the fluorescence increase in the sample.

30 In Fig. 2 typical time-dependent fluorescence curves obtained by switching the pH to 8 are shown. Fluorescein particles covered by layers of different thickness (9, 13, 15, and 18 layers) are compared with the control demonstrating the dissolving of naked fluorescein particles.

WO 02/017888

PCT/EP01/09908

- 23 -

As shown in Figure 3, after a comparatively short induction period (1) the rate of dissolving becomes constant (2) before finally the fluorescence in the bulk levels off (3). The initially more slowly increasing fluorescence is related to the start of core dissolving. At this stage of the process the structure of the polyelectrolyte multilayer may change because of the nascent osmotic pressure coming from dissolved fluorescein molecules. Shortly after the beginning of core dissolution the concentration of fluorescein inside the capsules becomes constant and almost saturated, since a steady state situation between progressing core dissolution and permeation is established. One may further assume a constant concentration gradient between the shell interior and the bulk because the bulk solution can be assumed as being infinitely diluted. Therefore the rate of fluorescein penetration through the polyelectrolyte layers to the bulk becomes constant. Indeed, a linear increase of the fluorescence is observed (2). This state corresponds to the stage of dissolution depicted in Figure 1E. The slope of the linear region decreases with the number of polyelectrolyte layers. Obviously an increasing number of adsorbed layers reduces the fluorescein penetration. After the core is completely dissolved, the fluorescein concentration inside the shell equilibrates with the bulk. The driving force for diffusion decreases and the release levels off (3).

Figure 4 shows the behaviour of the permeability times thickness as a function of the numbers of layers.

Example 2: Permeability as function of pH

Sodium poly(styrene sulfonate) (Na-PSS, MW ~ 70 000), poly(allylamine hydrochloride) (PAH, MW ~ 50 000), dextran (MW ~ 75 000 and 4 000 000), and bovine serum albumin all labeled with fluorescein isothiocyanate (FITC) were obtained from Aldrich. Hollow polyelectrolyte capsules were fabricated at pH = 7 by alternating adsorption of 8 layers PSS/PAH onto MF-particles of a diameter of 5.2 μm (microparticles GmbH, Berlin), using

WO 02/017888

PCT/EP01/09908

- 24 -

the filtration protocol (15). The cores were dissolved at pH = 1. Washings in 50 mM NaCl followed. Confocal images were taken by means of a confocal laser scanning microscope (TCS Leica). The excitation wavelength was 488 nm. The capsules were suspended into the FITC labeled polymer solution of a concentration of 1 mg/ml.

The exclusion properties of hollow polyelectrolyte capsules composed of 8 PSS/PAH layers templated on 5.2 μm melamin formaldehyde (MF) - particles for FITC-labeled dextran (with MW about 75 000) have been studied as a function of pH. Fig.5 (left) provides a confocal image of capsules in the presence of FITC-dextran at pH = 10. The interior of the capsules remains dark, while the background is fluorescent. This proves that at this condition the capsule wall is not permeable for FITC-dextran. Even deformed capsules do not reveal any fluorescence inside. However, at pH = 3 the capsule interior becomes as fluorescent as the bulk (Fig.5, center). This can only be explained by opening of the capsules for FITC-dextran at this low pH value.

It has to be mentioned that the "open" and "closed" states at relatively low pH and high pH, respectively, were observed for more than 90% of the capsules. The open state for FITC-dextran was observed for pH values up to 6. From pH 8 onwards most of the capsules are closed. At a pH value in between, i.e. pH = 7, open and closed capsules were simultaneously present.

The possibility of loading is demonstrated in Fig.5 (right) where the capsules were initially exposed to a FITC-dextran solution at pH = 3. Then the pH was shifted to 10 and the rest of FITC-dextran was removed from the bulk by centrifugation. It is remarkable that the capsules remain filled with fluorescent material as shown by the fluorescence profile through the confocal image. The interior of the capsule observes a bright and constant over time fluorescence while there is no fluorescence signal from solution.

WO 02/017888

PCT/EP01/09908

- 25 -

Similar experiments of changing the capsule wall permeability by pH changes and subsequent capsule loading were performed with FITC-dextran of a MW of 2 000 000 and FITC-labeled bovine serum albumin. The results are analogous to those with FITC-dextran of MW 75 000, but the pH value of the transition between the closed and open state differs by 1-1.5 pH units.

Example 3: Controlling capsule wall permeability by the salt concentration

- Two different types of polyelectrolyte capsules were used for these permeability experiments. 8 layers of sodium polystyrenesulfonate PSS and polyallylamine PAH were adsorbed on monodisperse templates of diameter 4.7 μm consisting of weakly polymerized melamine-formaldehyde resin. In one batch, core dissolution was conducted at pH 1 and the subsequent washings were performed in the presence of 0.05 M NaCl. This protocol yielded crumpled capsules (Fig. 6a). These capsules were covered by two additional layers and subsequently washed with Millipore water ($\sigma < 18 \text{ M}\Omega \text{ cm}$). After this treatment, the capsules assumed a spherical shape.
- The cores in the second batch were dissolved in 0.1 M HCl and washed afterwards with Millipore water thus avoiding the addition of salt. The latter protocol yielded homogeneous population of capsules with a spherical shape (Fig. 6b). The permeability behavior of the two types of capsules was similar. Essentially the results obtained with the second type of capsules will be reported in this example. Only in case of remarkable differences results obtained with capsules having additional two layers will be mentioned.

The permeability of the capsule walls was investigated by means of fluorescence labeled polymers polystyrenesulfonate PSS (120 000 g/mol), polyallylamine PAH (70 000 g/mol), dextrane (55 000 g/mol) and human albumine (70 000 g/mol). Polyallylamine PAH was used which was labeled

WO 02/017888

PCT/EP01/09908

- 26 -

at every 245th position with rhodamine B. In the Förster resonance energy transfer measurements, polyallylamine PAH labeled at every 120th position with fluoresceine was used. The capsules were added to a 5×10^{-3} M solution of the respective probe polymers, which concentration is always expressed in monomer units. The polymer permeation was followed by means of confocal imaging (TCSCN Leica, Germany). The amount of fluorescent molecules inside and outside the capsules was quantified.

The image in Fig.6b shows a confocal scan through the equatorial plane of the capsules, which were incubated with the labeled polymer solution for 24 hours. About 90% of the capsules excluded the polymers. The few with polymer filled capsules were broken and observed large holes through which the polymers could have diffused almost instantaneously into the capsule interior.

A similar experiment was then performed in the presence of 0.1 M NaCl. The confocal image (Fig. 6c) shows, that the interior of all capsules contains approximately the same concentration of the polymer as present in the bulk. This finding is consistent with an increase of the permeability of the polyelectrolyte wall for polymers in the presence of salt. This "wall opening" was observed for positively and negatively charged polymers as well as for the dextrane representing a neutral polymer. Hence, salt induced changes in the structure of the probe polyelectrolytes, such as coiling of the charged species cannot be the responsible mechanism for the penetration. Rather salt-induced changes of the structure and properties of the polyelectrolyte complexes in the wall have to be considered as the cause for the strong increase in permeability. As evident from the images, the fluorescent probe molecules were accumulated on the surface or inside the capsule wall. This was expected for PSS, which is adsorbed onto the positively charged capsule surface, but either for the labeled PAH an exchange of PAH molecules in the capsule wall or an adsorption to the inner PSS layer has to be assumed.

WO 02/017888

PCT/EP01/09908

- 27 -

The permeability was next investigated in dependence on the salt concentration. The capsules were mixed with 5×10^{-3} M PAH-Rhodamine and increasing concentrations of sodium chloride. The dispersions were incubated for 10 min and the amount of polymer in the interior was compared with the polymer concentration in the bulk. Up to 5×10^{-3} M NaCl, no polymer penetration into the capsules was detected, but for higher concentrations, the capsules became increasingly permeable for PAH-Rh. At 2×10^{-2} M NaCl, the time of PAH-Rh penetration was estimated by means of confocal imaging overtime. The time between salt addition and complete equilibration of the polymer concentration inside and outside the capsules was app. 6 min for capsules consisting of 8 layers. A slower penetration occurring within 20 min was measured for capsules of 10 layers. Confocal imaging could not follow the kinetics at higher concentrations, because the diffusion and equilibration of the polymer concentration was too fast.

The minimum amount of salt for inducing the polymer penetration was determined by incubating the capsules in polymer solution, at various NaCl concentrations for 24 hours. Then, the percentage of filled capsules was determined by counting in the confocal images the number of filled and empty capsules for app. 100 capsules each (Table 1). While in the 10^{-2} M salt solution all capsules contained labeled polymer, in the 0.5×10^{-2} M salt solution only 16% of the capsules appeared to be filled within 24 h. In still lower NaCl, the capsules remained impermeable for the polymer. Hence, the permeability increase of the capsules for macromolecules occurred within a rather narrow range of salt concentration between 0.5 and 2×10^{-2} M.

It was further examined, whether the rate limiting step of permeation after the addition of salt is provided by salt-induced structure changes in the capsule wall making the wall more permeable or by the diffusion of the polymer through the layer. For this the permeation of PAH $M_n = 70\ 000$

WO 02/017888

PCT/EP01/09908

- 28 -

was observed either immediately after the simultaneous addition of 10^{-2} M salt (NaCl) and 5×10^{-3} M PAH or the sample was incubated for 12 hours in 10^{-2} M NaCl and the PAH was added only then. In the second case, it was found that the characteristic permeation time was about 30 min, which was smaller than the characteristic permeation time of about 50 min observed when NaCl and the polymer were added at the same time. These numbers are two fold remarkable. They demonstrate that 1) the permeation of the polymer under the observed conditions is overall slow requiring at least 30 min for concentration equilibration, and 2) that the permeability induction by salt itself requires several minutes.

Regarding the mechanism of the polymer permeation through the capsule wall two extreme situations may be distinguished. Either incubation in the salt solution led to the formation of water-filled pores through the wall across which the polymers can diffuse into and out of the capsule, or the presence of ions in the solution weakens the electrostatic binding between PSS and PAH allowing for diffusion of the polymer through the network via transient bond breaking and re-establishing. Pore formation either induced by pH or by salt was observed for macroscopic flat films composed of weak polyelectrolytes. These relatively large pores had a radius in the order of 10^2 nm. SFM was thus applied to polyelectrolyte capsules either prepared by drying a capsule dispersion in H_2O or in 0.1 M NaCl applied onto a mica substrate. However, except the presence of some salt crystals no differences in the capsule wall topology were observed. This result argued the formation of large pores as the cause of the permeability increase.

Assuming that the polymer penetration occurs via diffusion through water-filled pores the apparent pore cross section is of the order of 1000 nm^2 . This would compare to either one pore of 17 nm radius or to 80 pores of 2 nm each. In any case, it is worth to note that the pore area would constitute a very tiny area fraction of the capsule surface only. From these

WO 02/017888

PCT/EP01/09908

- 29 -

considerations, it becomes clear that with SFM it is hardly possible to detect these pores even if their existence is sure.

In order to find changes in the capsule wall morphology in dependence on the salt concentration of the bulk electrolyte, Förster resonance energy transfer FRET between fluorescent labeled polyelectrolytes was applied. The capsules were covered by addition of six polyelectrolyte layers in the order PSS, PAH-fluoresceine, PSS, PAH-rhodamine, PSS, PAH. The polymers were adsorbed in presence of 0.5 M NaCl, but afterwards the capsules were extensively washed by water. FRET of the capsule dispersion was measured 5 min after adding NaCl solutions of different concentrations. Excitation was set at 495 nm, where only fluoresceine absorbs light. If rhodamine molecules are located near the fluoresceine, (up to 6 nm) resonance energy transfer takes place and one can observe the rhodamine fluorescence, too (Fig. 7, inset).

The relative transfer efficiency E_{FRET} was defined as $E_{\text{FRET}} = (I_{\text{rh}} - 0.4I_{\text{fi}}) / (0.6I_{\text{fi}} + I_{\text{rh}})$ where I_{fi} is the fluorescence intensity at 522 nm corresponding to the maximum in fluoresceine emission and I_{rh} is the intensity at 582 nm, corresponding to the rhodamine emission.

The factor 0.4 takes into account the fluorescence of the donor at the acceptor emission wavelength. The plot of E_{FRET} against salt concentration shows a clear decrease in the transfer efficiency (Fig. 7), occurring exactly in the concentration range where the capsule wall becomes permeable for PAH. At higher salt concentrations, the E_{FRET} kept almost constant. Hence, it can be concluded that FRET reported remarkable changes in the topology of the polyelectrolyte wall occurring within a narrow salt concentration range around 10^{-2} mol/L. The decrease of E_{FRET} with the salt concentration indicates an increase of the distance between the dye molecules of rhodamine and fluoresceine. The FRET signal was completely restored within 5 min when the capsules were expose to water again. These

WO 02/017888

PCT/EP01/09908

- 30 -

findings are obviously consistent with some reversible swelling of the polyelectrolyte layer.

5 The correlation between the permeability increase of the wall opening and FRET decrease allowed for characterizing the salt-induced capsule wall changes in the absence of a permeating polymer. The latter may have had some influence on the wall properties, especially as much remarkable amounts of the permeating polymer adsorbed to the wall. The capsules were introduced in 1.5×10^{-2} M NaCl and the E_{FRET} was measured in
10 dependence on the time (Fig. 8). After 20 min the FRET was saturated at a value of $E_{\text{FRET}} = 0.31$. The time scale of the changes was consistent with the permeability change observed by means of confocal microscopy. Both, the time and the degree of the FRET signal change related to wall structure changes increased with the salt concentration.

15 The mechanism and driving forces causing both layer permeability increase and the decrease of the fluorescence energy transfer occurring in a narrow salt concentration range are discussed below. The rather slow permeation indicates, if water-filled pores are assumed as the basic pathway for
20 permeation, a very small pore area. On the other hand, the change of the FRET signal can only be understood assuming conformational changes affecting the majority of the labeled polymers. Hence, salt induces layer changes throughout. This is less constant with assuming the much-localized formation of a small number of pores. Therefore we interpret the
25 permeability increase as a result of an increased solubility or interactions of the permeability species with the layer polymers, which are caused by an increased salt concentration.

30 A higher salt concentration was found to soften the structure of the layer because the ion pairs found between the polyanions and the polycations may partly open, because the free ions available in the bulk would screen the charges in the layer. Layer swelling can thus be understood as a

WO 02/017888

PCT/EP01/09908

- 31 -

molecular elasticity relaxation of the polymer pairs, leading to a more bulky arrangement of the layer polymers. It is worth to note that at 10^{-2} M NaCl the Debye screening length is just 3 nm comparing well to a layer thickness increment of a polyanion/polycation pair. At lower electrolyte concentrations, the electrostatic interactions have a larger range providing an electrostatic interaction over the whole layer.

An important issue for many applications is the reversibility of the permeability switching process. The fact, that the capsules were prepared at high salt concentrations and are nevertheless impermeable for the macromolecules after washing demonstrates the reversibility of the capsule opening and closing. In further experiments the time of closing the capsule wall was determined. A 3×10^{-2} M NaCl solution was applied for 30 min to the capsules. Afterwards they were diluted into pure water to app. 5×10^{-3} M NaCl. After ten minutes, the capsules became impermeable for the PAH-Rhodamine as was observed by means of confocal imaging. The FRET signal was recovered within 5 min (Fig. 8).

The reversibility of the wall permeability change for polyelectrolytes allows for an easy and soft encapsulation of macromolecules, such as polypeptides, DNA, enzymes, or polymers avoiding any chemical stress caused by aggressive substances, solvents, pH, or heat applied during encapsulation. However, loading of capsules with macromolecules at high salt concentration following by washing with water yielded only small amounts of encapsulated polymer in the interior. Probably, the macromolecules were washed out faster than the wall closed.

A much higher efficiency of loading was however achieved when the polymer for encapsulation was present in the bulk during closure of the wall induced by salt removal. (Fig. 10). By this way, the capsules were loaded with a 5×10^{-3} M PAH-Rh solution (Fig. 9). Taking the fluorescence intensity of the confocal image of the PAH-Rh solution as standard, the

WO 02/017888

PCT/EP01/09908

- 32 -

amount of encapsulated polymer could be determined. The average of the interior PAH-rho concentration was about 50% of that of the initial solution.

5 Another important property of the loaded capsules is their release behavior. The loaded capsules were incubated for 30 min either in 2 ml water or in 2 ml of a 3×10^{-2} M salt solution. The capsules were removed from the sample by centrifugation and the concentration of the PAH-Rh in both solutions was determined by fluorescence spectroscopy.

10 The dependence of the rhodamine emission on the salt concentration, was taken into account by means of adjusting the salt free solution afterwards to 3×10^{-2} M before the measurement were performed.

15 While in the pure water the amount of released PAH could not be detected almost all encapsulated PAH-Rh was released in 3×10^{-2} M NaCl. Confocal image proved that the interior of those capsules was void of fluorescent polymer again.

20 Example 4: Temperature dependent permeability

Monodisperse spheres ($d = 5.94 \mu\text{m}$) of a weakly polymerized melamine-formaldehyde resin were used as templates (Microparticle GmbH). The particles were covered by alternating adsorption of sodium polystyrene sulfonate PSS (MW 70 000 g mol^{-1} , Aldrich) and polyallylamine hydrochloride PAH (MW 70 000 g mol^{-1} , Aldrich) from an aqueous solution of 10^{-2} M PSS in 0.1 M sodium chloride. After 8 layers the core was dissolved in 0.1 M hydrochloric acid. The decomposition products were removed by washing with acid and an aqueous solution of 10^{-2} M NaCl.

25 Hollow polyelectrolyte capsules remained. In order to remove some defects in the capsules wall caused by mechanical stress during the dissolution two additional layers were coated on the capsules after the dissolution of

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WO 02/017888

PCT/EP01/09908

- 33 -

the cores. Afterwards the capsules were washed extensively (8 times) with Milli-Q-water (resistance $>18 \text{ M}\Omega \text{ cm}^{-1}$). By this procedure, traces from salt and protons were essentially removed and a pH value of 6 was reached.

5 For the study of the permeability, fluorescence labeled polymers were used. PAH (MW 70 000, 1 rhodamine molecule per 245 PAH units and MW 15 000, 1 rhodamine per 320 PAH units) and PEI (MW 2 000, 1 fluoresceine molecule per 650 PEI units) were labeled by reaction with
10 rhodamine isothiocyanate or fluoresceine isothiocyanate. Labeled PSS (1 rhodamine molecule per 600 polymer units) was synthesized by copolymerization of styrene sulfonate with methacroyloxyethyl thiocarbamoyl rhodamine B (Polyscience). The MW of the polymer was determined being 120 000 g/mol by gel permeation chromatography.
15 Fluoresceine labeled human serum albumine (MW 69 000) and rhodamine labeled dextrane (MW 40 000 g/mol) were purchased from Aldrich company.

Laser scanning microscopy was performed for the determination of the
20 loading grade of the capsules. An inverse research microscope (Leica, Germany) with either a 40 x or a 100 x oil immersions objective was used. The amounts of polymer/monomer in the interior were determined by integration of the fluorescence intensity inside the capsules. The exact concentration was taken from calibration curves, determined for each
25 fluorescent probe separately. Due to the broad distribution of permeability for the capsules, statistical values were taken from 5 capsules each. Capsules, which could be recognized clearly as broken once (appr. 20%), were excluded from the determination. The temperature dependent measurements were done with an home-made aluminium device on top of
30 the sample, by which the temperature was settled by an electronic controller.

WO 02/017888

PCT/EP01/09908

- 34 -

Scanning force microscopy was applied to capsules, dried on air on a mica substrate. The drying process after the heat treatment at 60°C was done by transferring a drop of the capsule solution to the mica surface and drying without any change of the temperature.

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4.1 Permeability

The permeability of the capsules was studied for positively (PAH) and negatively charged (PSS), zwitterionic (albumine) and noncharged (dextran) fluorescent polymers (dextran). In general, the capsules were mixed with the probe polymers in a concentration of about 2×10^{-3} mol/L with respect to the polymer units and incubated for 15 min. Then, the fluorescence intensity in the center of the capsules was measured by confocal imaging. The fluorescence events within the cross section of the capsule were integrated and compared with the fluorescence events in the bulk solution. Less fluorescence intensity inside than outside the capsules indicates an impermeability of the capsule wall for the probe polymer. Equal fluorescence intensities inside and outside of the capsules at the start of the experiment is caused by fast penetration of the polymers through the capsule wall, mostly observed for broken capsules. In case of dextrane as described below the fluorescence inside the capsules is higher than in the bulk phase indicating an accumulation of the polymer in the interior.

First the permeability of the capsule wall for the probe polymers was studied before any heat treatment. Within 24 hours the majority of capsules were impermeable for charged polyelectrolytes of MW above 60 000 g/mol independent on the sign of the charge. Figure 11a shows the confocal image for PAH of 70 000 g/mol. In the case of uncharged dextrane an unusual accumulation of the polymer inside of appr. 20% of the capsules was observed (Fig. 11b), while the other capsules exclude the polymer, too.

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WO 02/017888

PCT/EP01/09908

- 35 -

Further influence of the length of the polymer chain on penetration was determined. While within 1 hour 80% of the capsules are impermeable for PAH 70 000 g/mol, about 65% were impermeable for PAH 15 000 g/mol and for PEI 2000 all capsules were permeable. Small dye molecules such as fluoresceine, rhodamine and acridine orange can penetrate the capsule wall independent of their charge.

The dependence of the permeability of capsule walls on temperature was followed online by confocal imaging (in situ) by heating capsules in 10° steps from room temperature to 80°C. At each step the temperature was allowed to stabilize for 5 min. The changes in the polymer distribution within the capsules and the bulk solution were similar for each polymer and are demonstrated for heating the capsules in presence of rhodamine labeled PSS. Up to 50°C the polymers was excluded from the capsule wall, but at higher temperature the PSS starts to flow into the capsules until an equilibrium or even a slight excess was reached inside the capsules. The rate of penetration increases with the temperature furthermore. This is a clear proof, that the capsule wall can be opened for large polymer molecules very easy by increase of the temperature.

It was further examined whether the opening is an irreversible process or if the capsule walls can be closed again by reducing the temperature below 50°C. This was investigated by treating the capsules in absence of polymers at different temperature conditions deviating in time of heating and cooling.

Possible mechanisms leading to an increase of capsule wall permeability are discussed below. Besides the fact, that diffusion through a membrane increases generally with temperature due to the higher mobility of the polymer species, the rather strong effect observed in a narrow temperature range indicates structural changes in the polyelectrolyte wall. One possible mechanism could be the opening of pores in the polyelectrolyte membrane

WO 02/017888

PCT/EP01/09908

- 36 -

as it is described above for treatment with pH and salt for similar systems. It was observed that PSS/PAH capsules shrink at higher temperature by more than 10% in diameter. The mechanical stress at such process could cause also the formation of pores. In order to find such pores, the capsule wall was investigated by high resolution Scanning Force Microscopy after drying capsules on a mica substrate. Three samples were prepared before, during, and after heating to 60°C, but significant differences were not observed apart from the reduction of the capsules in diameter. Despite of this experimental result, the formation of pores can not be fully excluded because of the subsequent drying process can change the capsule structure remarkably.

Another mechanism is the weakening of the electrostatic bounds between polyanions and polycations. This leads to an increased charge density in the film, connected with stronger hydration, swelling and a better diffusion of the polymers through the wall. It was tried to support this mechanism by investigations of the resonance energy transfer in capsules consisting of 14 layers, in which the 10th layer is PAH-fluoresceine and the 12th layer is PAH-rhodamine. The spectra of the solution were taken before, during, and after heating to 60°C for 20 min. The spectra before and after the heating look quite similar, indicating an almost reversible change in the wall structure. The spectrum at 60°C deviates remarkably from the other spectra. The decrease of the rhodamine fluorescence indicates an increase of the averaged distance between fluoresceine and rhodamine molecules or the polymer layers, respectively. In order to exclude simple heating effects as origin of the observed spectra changes, a PAH polymer containing on every 106th position a fluoresceine molecule and on every 345th position a rhodamine molecule (MW 70 000 g/mol) was investigated under identical conditions.

The findings offer a unique possibility to encapsulate macromolecules, since the permeability of capsule walls was found to be controlled by

WO 02/017888

PCT/EP01/09908

- 37 -

temperature: the capsule wall is opened by heating and surrounding material can stream in. If the capsules are filled with the desired materials the wall can be closed again by cooling down the solution. Afterwards the remaining materials outside, e.g. polymers can be washed away and the enclosed macromolecules remain inside the capsules. The encapsulation was done successfully for all labeled polymers by heating impermeable capsules (Fig. 12a) to 60°C for 20 min. After cooling and washing, the capsules contained the polymer as shown for example in Figure 12b. A quantitative determination of the fluorescence intensity of the original polymer solution and the encapsulated polymer in the interior yielded a 30% of the bulk concentration in the interior. Both probe polymers used, i.e. labeled PSS as well as PAH were captured in the interior indicating a high reversibility of the permeability. Even storage of the filled capsules for more than one month lead only to some loss of polymer. This loss was especially strong with more than 50% for PAH, but zero in case of dextrane.

WO 02/017888

PCT/EP01/09908

- 38 -

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WO 02/017888

PCT/EP01/09908

- 40 -

Table 1: Percentage of filled capsules after 24 hours incubation time in solutions of PAH-rho, 5×10^{-3} M and in presence of different salt concentrations.

Salt concentration / 10^{-4} mol/L	1	5	10	50	100	200
Percentage of filled capsules/ %	4.6	8.5	6	16.7	100	100

WO 02/017888

PCT/EP01/09808

- 41 -

Claims

1. A process for controlling the permeability of polyelectrolyte
5 multilayer capsules, wherein at least one of the parameters of pH, temperature, light, salt concentration, ion composition, ion concentration, ionic strength, solvent composition or solvent concentration is adjusted or/and varied during preparation, storage or/and use of the capsules.
- 10 2. A process for controlling the permeability of polyelectrolyte multilayer capsules, wherein the number of polyelectrolyte layers or/and chemical composition, hydrophobicity, polarity, stiffness, molecular weight, the charge or charge density of layer constituents,
15 in particular polyelectrolytes is adjusted in such a way that the desired permeability is achieved.
3. A process according to claim 1, wherein a pH of at least 8 is
20 adjusted to render the polyelectrolyte multilayer capsules impermeable.
4. A process according to claim 1, wherein a pH of less than 6 is
adjusted to render the polyelectrolyte multilayer capsules permeable.
- 25 5. A process according to any one of the preceding claims, wherein a salt concentration of at least 2×10^{-2} M is adjusted.
6. A process according to any one of claims 1-4, wherein a salt
concentration of less than 5×10^{-3} M is adjusted.
- 30 7. A process according to any one of the preceding claims, wherein the temperature is set to at least 50°C.

WO 02/017888

PCT/EP01/09908

- 42 -

8. A process according to any one of claims 1-7, wherein the temperature is set to less than 50°C.
9. A process according to any one of the preceding claims, wherein a solvent composition containing an alcohol and/or water is used.
10. A process according to claim 2, wherein the number of polyelectrolyte layers is at least 8.
11. A process according to any one of the preceding claims, wherein lipids, surfactants, dyes, drug molecules, nanoparticles or/and biopolymers, such as polysaccharides, polypeptides, nucleic acids are included as layer constituents.

WO 02/017888

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PCT/EP01/09908

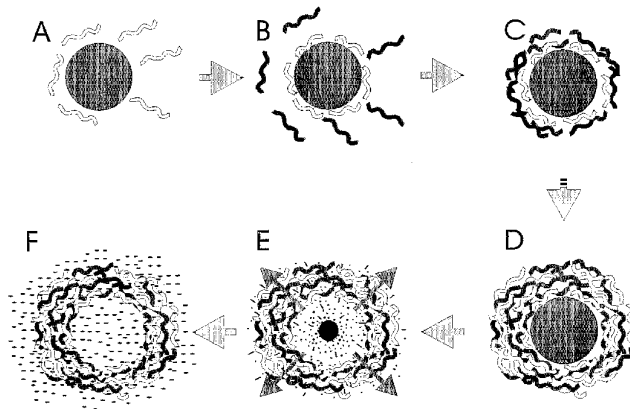


Fig. 1

WO 02/017888

PCT/EP01/09908

2/16

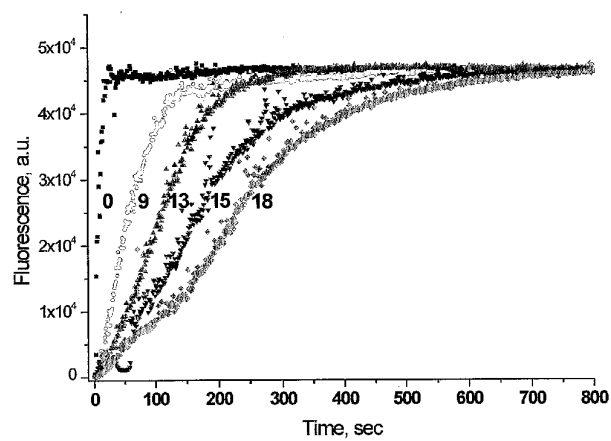


Fig. 2

WO 02/017888

PCT/EP01/09908

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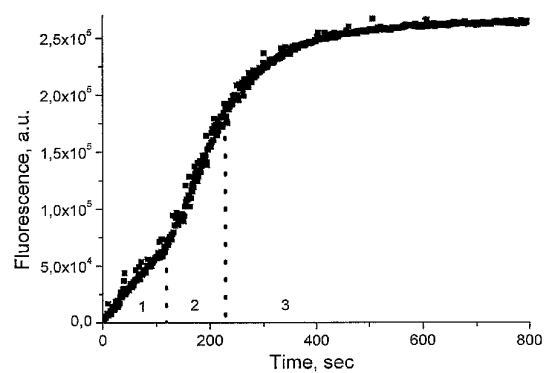


Fig. 3

WO 02/017888

PCT/EP01/09908

4/16

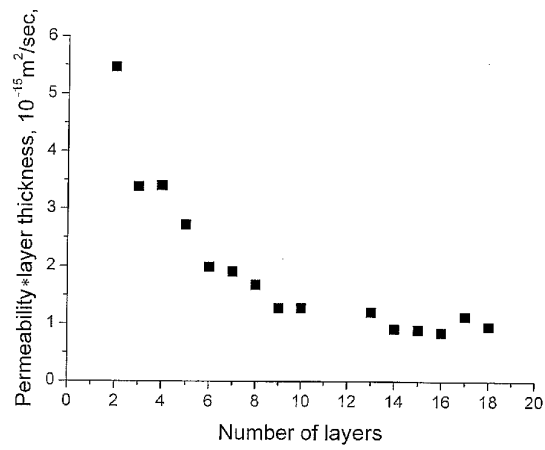


Fig. 4

WO 02/017888

PCT/EP01/09908

5/1 6

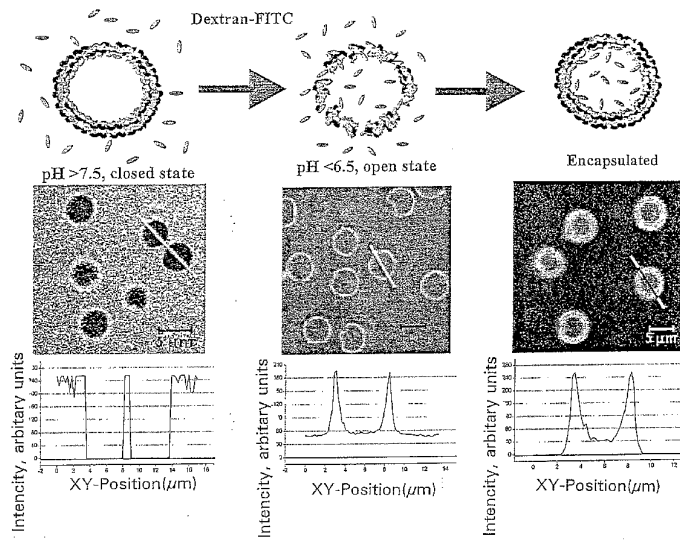


Fig. 5

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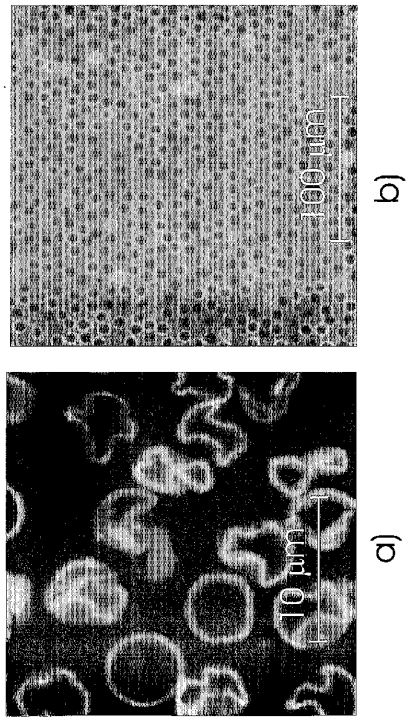
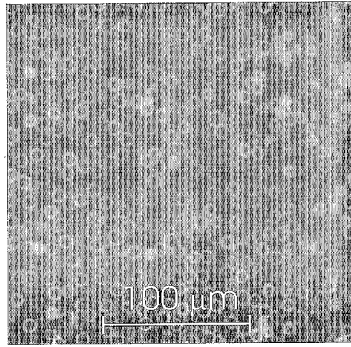


FIG. 6

WO 02/017888

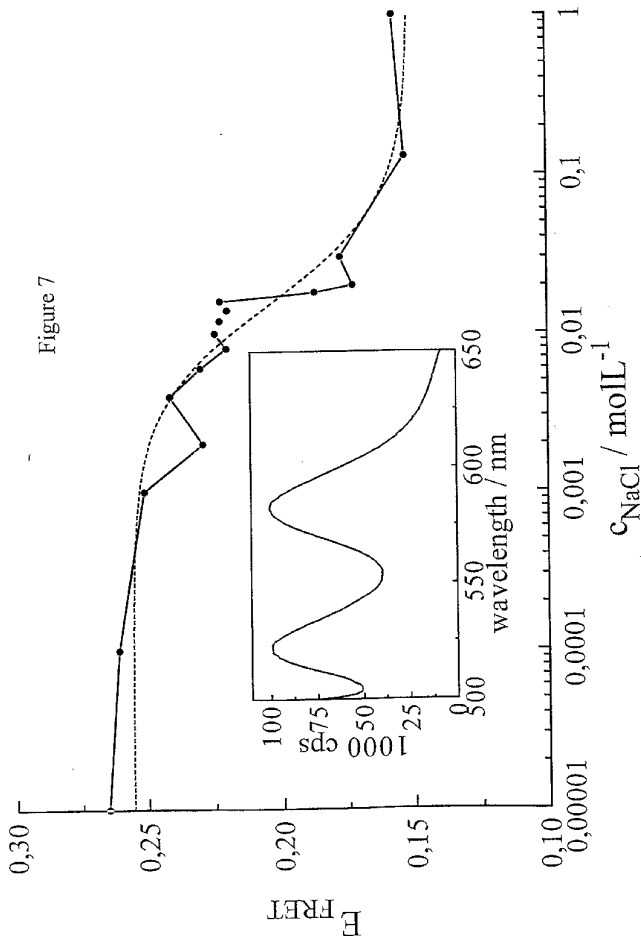
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PCT/EP01/09908



c)

FIG. 6



WO 02/017888

PCT/EP01/09908

9/16

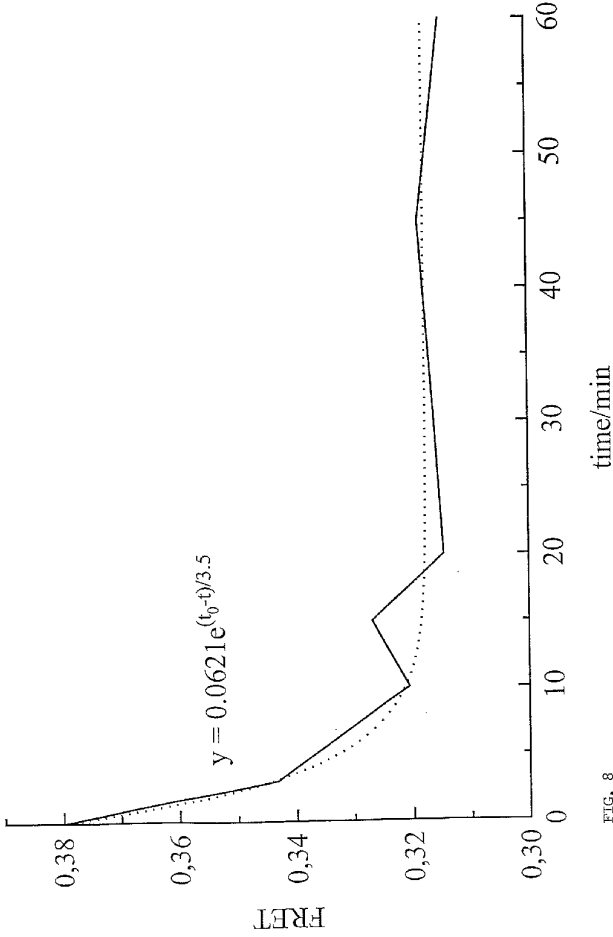


FIG. 8

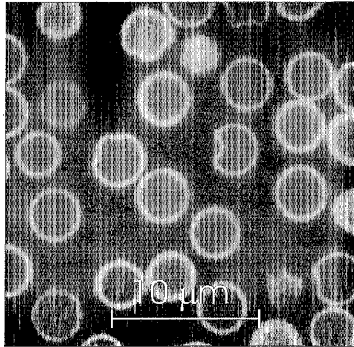


Figure 9

WO 02/017888

11/16

PCT/EP01/09908

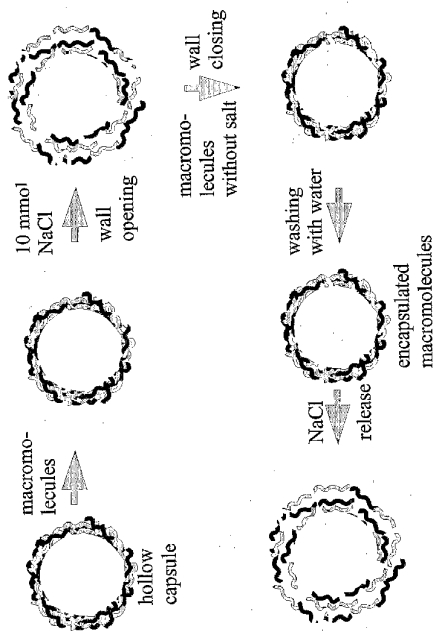


Fig. 10

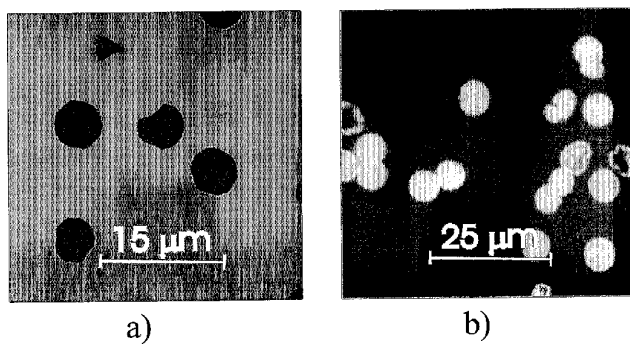


Figure 11

WO 02/017888

13/16

PCT/EP01/09908

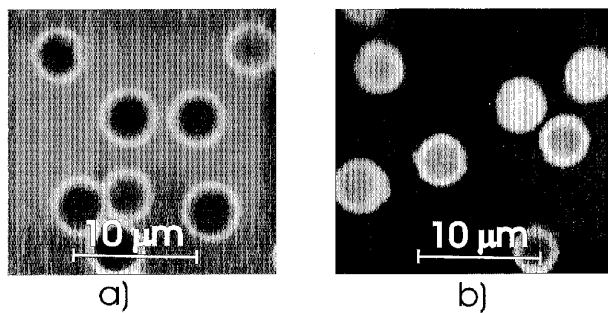


Fig. 12

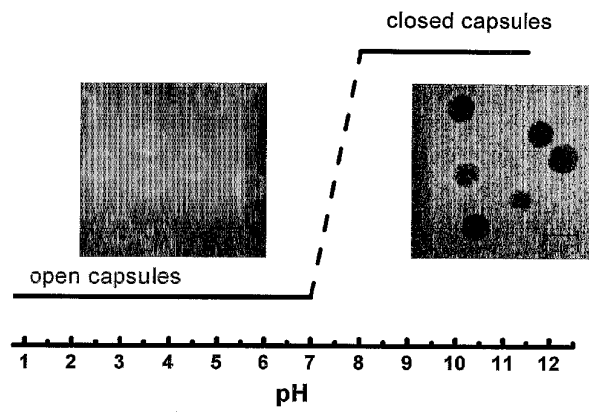


Fig. 13

WO 02/017888

15/16

PCT/EP01/09908

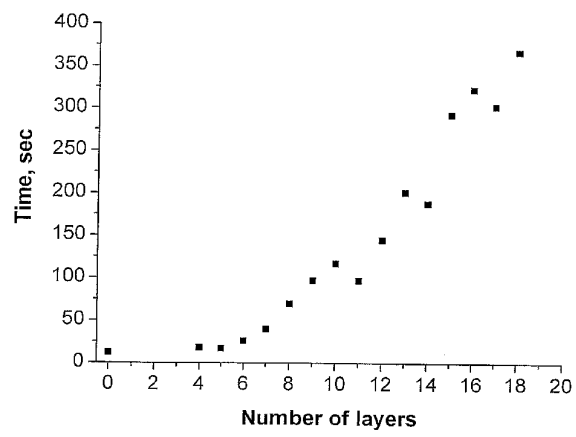
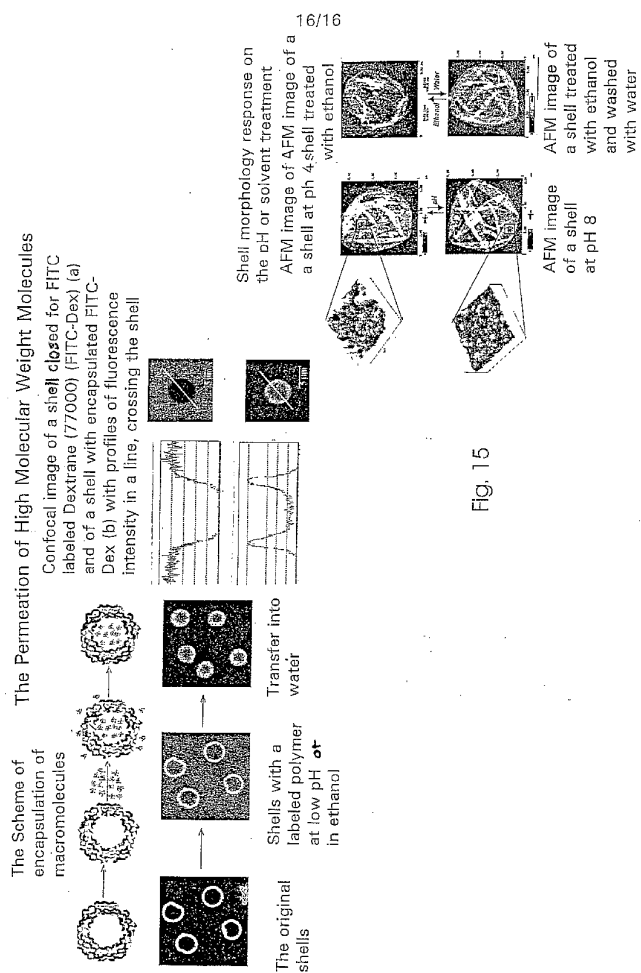


Fig. 14

WO 02/017888

PCT/EP01/09908



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【国際調査報告】

INTERNATIONAL SEARCH REPORT		International Application No. PCT/EP 01/09908
A. CLASSIFICATION OF SUBJECT MATTER IPC 7 A61K9/52 B01J13/02		
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) IPC 7 A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) WPI Data, PAJ, EPO-Internal, BIOSIS		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
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E	WO 02 09865 A (MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V.) 7 February 2002 (2002-02-07) page 1, line 1 - line 18 page 7, line 24 - line 32 page 11, line 29 - page 12, line 13	1,2
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-/-		
☒ Further documents are listed in the continuation of box C. ☒ Patent family members are listed in annex.		
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Date of the actual completion of the international search 23 May 2002		Date of mailing of the international search report 03/06/2002
Name and mailing address of the ISA European Patent Office, P.B. 5016 Patentstr. 2 NL - 2209 HV Rijswijk Tel (+31-70) 340-2040, Tx 31 651 epo nl, Fax (+31-70) 340-3016		Authorized officer Benz, K

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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フロントページの続き

(74)代理人 100114890

弁理士 アインゼル・フェリックス＝ラインハルト

(74)代理人 230100044

弁護士 ラインハルト・アインゼル

(72)発明者 アレクセイ アンティポフ

ドイツ連邦共和国 ゴルム イン デア フェルトマルク 7

(72)発明者 エウリディス ヴィエイラ

ドイツ連邦共和国 ポツダム ケア オヴ マックス＝ブランク＝インスティトゥート フュア
コロイド＝ウント グレンツフレッシェンフォルシュング

(72)発明者 ジェマ イバルス

ドイツ連邦共和国 ポツダム ケア オヴ マックス＝ブランク＝インスティトゥート フュア
コロイド＝ウント グレンツフレッシェンフォルシュング

(72)発明者 グレーブ スホルコフ

ドイツ連邦共和国 ポツダム フォイアーバッハシュトラッセ 3 5

(72)発明者 ラルス デーネ

ドイツ連邦共和国 ベルリン シュティラーツァイレ 3

(72)発明者 チャンユー ガウ

ドイツ連邦共和国 ゴルム アム ミューレンベルク 2

(72)発明者 エドウィン ドナート

ドイツ連邦共和国 ギーゼンホルスト ドレーツァー シュトラッセ 1

(72)発明者 ヘルムート メーヴァルト

ドイツ連邦共和国 ビンゲン ドクトル＝ゲバウアー＝シュトラッセ 2 1

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