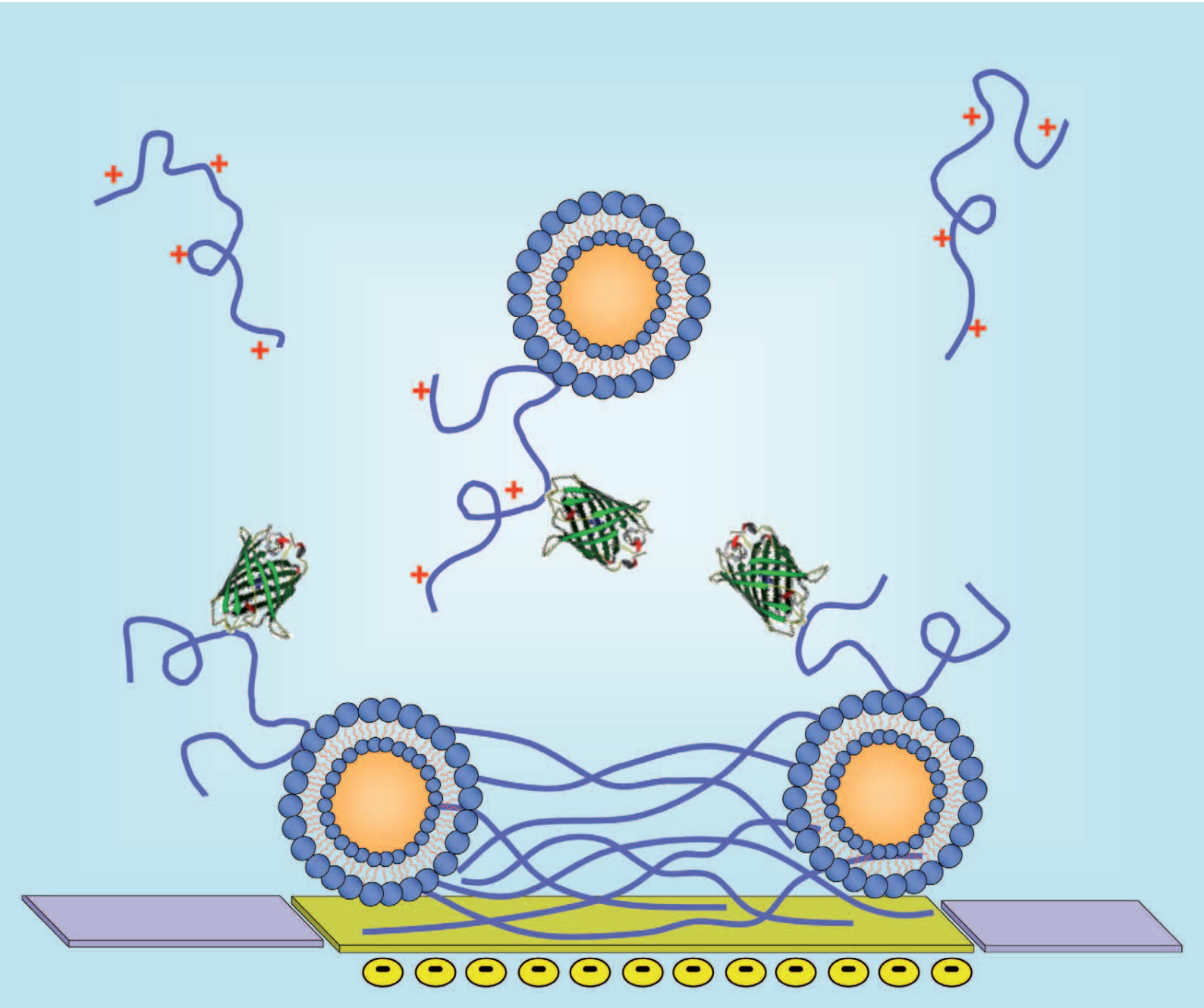
Soft Matter

www.softmatter.org

Volume 3 | Number 5 | 7 May 2007 | Pages 497–652



ISSN 1744-683X

RSC Publishing

Chitosan: a soft interconnect for hierarchical assembly of nano-scale components

Gregory F. Payne*[a] and Srinivasa R. Raghavan*[b]

Received 26th September 2006, Accepted 28th November 2006
First published as an Advance Article on the web 16th January 2007
DOI: 10.1039/b613872a

Traditional microfabrication has tremendous capabilities for imparting order to hard materials (*e.g.*, silicon wafers) over a range of length scales. However, conventional microfabrication does not provide the means to assemble pre-formed nano-scale components into higher-ordered structures. We believe the aminopolysaccharide chitosan possesses a unique set of properties that enable it to serve as a *length-scale interconnect* for the hierarchical assembly of nano-scale components into macro-scale systems. The primary amines (atomic length scale) of the glucosamine repeating units (molecular length scale) provide sites to connect pre-formed or self-assembled nano-scale components to the polysaccharide backbone (macromolecular length scale). Connections to the backbone can be formed by exploiting the electrostatic, nucleophilic, or metal-binding capabilities of the glucosamine residues. Chitosan's film-forming properties provide the means for assembly at micron-to-centimetre lengths (supramolecular length scales). In addition to interconnecting length scales, chitosan's capabilities may also be uniquely-suited as a *soft component–hard device interconnect*. In particular, chitosan's film formation can be induced under mild aqueous conditions in response to localized electrical signals that can be imposed from microfabricated surfaces. This capability allows chitosan to assemble soft nano-scale components (*e.g.*, proteins, vesicles, and virus particles) at specific electrode addresses on chips and in microfluidic devices. Thus, we envision the potential that chitosan may emerge as an integral material for soft matter (bio)fabrication.

[a]*Center for Biosystems Research, University of Maryland Biotechnology Institute, 5115 Plant Sciences Building, College Park, MD 20742, USA. E-mail: payne@umbi.umd.edu; Fax: +1 301 314 9075; Tel: +1 301 405 8389*
[b]*Department of Chemical and Biomolecular Engineering, University of Maryland, College Park, MD 20742, USA. E-mail: sraghava@eng.umd.edu; Fax: +1 301 405 0523; Tel: +1 301 405 8164*

Why a soft interconnect?

Microfabrication is the technology that has demonstrated the greatest capability for creating ordered structures over length scales that span from the submicron to the centimetre. Yet microfabricators are facing an interesting dilemma in their quest to fabricate chips capable of continuing the exponential

Gregory F. Payne

Gregory F. Payne received his B.S. and M.S. degrees in Chemical Engineering from Cornell University in 1979 and 1981, respectively. He received his Ph.D. in Chemical Engineering from The University of Michigan in 1984. After completing his Ph.D., he returned to Cornell to do post-doctoral work with Michael Shuler in biochemical engineering. In 1986 Dr Payne joined the faculty with joint appointments in Chemical and Biochemical Engineering at the University of Maryland Baltimore County (UMBC) and in the Center for Agricultural Biotechnology (now the Center for Biosystems Research) at the University of Maryland Biotechnology Institute (UMBI). His research is focused on biofabrication—the use of biological or biomimetic materials and processes for construction. Specifically, his group biofabricates using enzymes and biologically-derived polymers such as chitosan.

Srinivasa R. Raghavan

Srinivasa R. Raghavan received his B.Tech. in Chemical Engineering from the Indian Institute of Technology, Madras in 1992 and graduated with a Ph.D. in Chemical Engineering from North Carolina State University in 1998. He performed post-doctoral research in Chemical Engineering at the University of Delaware after which he joined the Department of Chemical and Biomolecular Engineering at the University of Maryland at College Park in 2001. Dr Raghavan's interests are in the broad area of complex fluids and nanomaterials, and his research group integrates a range of physical, chemical and biological methods to prepare and analyze materials that have structure over a range of length scales.

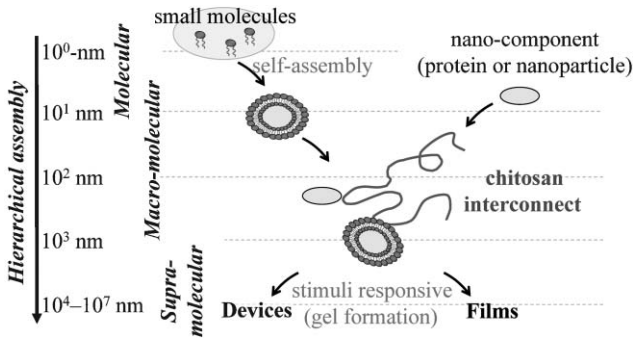


Fig. 1 Chitosan as a length-scale interconnect for hierarchical assembly. The figure shows that nano-scale assemblies of small molecules (*e.g.*, a vesicle) or other nano-components (*e.g.*, a protein or nanoparticle) can be connected to sites along a chitosan chain. These structures, in turn, can be connected into larger supramolecular assemblies and deposited at specific device addresses or formed as free-standing films.

increase in computer power. At the same time that conventional microfabrication is struggling to economically fabricate below 60 nm, there is an emergence of various nano-scale components (*e.g.*, carbon nanotubes and quantum dots). Yet hierarchical methods to connect these discrete, pre-formed nano-scale components into macro-scale systems are not available.

Compared to the fabrication of silicon wafers, the creation of ordered soft devices is far less advanced. Yet fabricators of soft devices will face the same challenge—how to connect pre-formed nano-components over a hierarchy of length scales. An additional challenge (or opportunity) is the connection of soft nano-scale components (*e.g.*, proteins, vesicles and virus particles) into hard devices for biosensor or microsystem applications.

We believe the aminopolysaccharide chitosan may emerge as an important material for soft matter fabrication because this biopolymer possesses a set of properties that uniquely equips it for hierarchical assembly of nano-scale components over a range of length scales. The potential of chitosan as a *length-scale interconnect* is illustrated in Fig. 1. As indicated, chitosan provides sites for connecting nano-scale components (10^0–10^1 nm) along its linear backbone (10^1–10^3 nm). Assembly at larger length scales occurs through chitosan's stimuli-responsive gel-forming properties that enable this polysaccharide to self-organize at the macro-scale ($>10^3$ nm). Importantly, chitosan films and gels can be induced to form in response to localized electrical stimuli. Thus, chitosan offers properties both to connect length scales and to connect nano-scale components to electronic devices. Chitosan's potential as a *soft component–hard device interconnect* was recently reviewed for biological systems.[1] Here, we describe our rationale for proposing chitosan as a length-scale interconnect for hierarchical assembly.

Molecular length scale (0.1–1 nm)

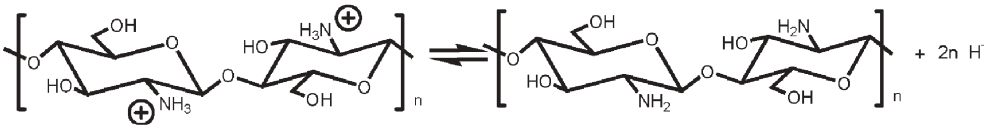
Chitosan is prepared by *N*-deacetylation of the linear β-(1→4)-linked polysaccharide chitin. Since chitin deacetylation is rarely complete, chitosan is formally a copolymer of *N*-acetyl-glucosamine (GlcNAc) and glucosamine (GlcN). The unique chemical feature of chitosan is the primary amine of the GlcN residues. As illustrated by the reaction below, these primary amines can be protonated conferring a positive charge to chitosan, and at a sufficiently high charge density, chitosan becomes soluble in aqueous solution. In fact, the term "chitosan" is an operational definition of an acid-soluble, deacetylated chitin derivative. While providing some information of the co-polymer composition (typically the degree of acetylation of chitosan is less than 50%), this operational definition provides no specification of chitosan's molecular weight or sequence (random or blocky).[2,3] As a result of this ambiguity, there can be considerable disagreement in the literature on the finer details of chitosan's structure and properties.

Chitosan's primary amines confer important material properties. At low pH, the amines are protonated making chitosan a *cationic polyelectrolyte*. This polyelectrolyte property has been employed by several groups to generate multilayer films or capsules using layer-by-layer assembly.[4–15] At high pH, the amines are deprotonated and chitosan undergoes a transition from a soluble cationic polyelectrolyte to an insoluble polymer. Importantly, this *pH-responsive* switch is near neutrality (chitosan's apparent pK_a has been reported to range between 6 and 7)[16–21] suggesting chitosan as a biologically-derived stimuli-responsive polymer for medical applications (*e.g.*, injectable matrices).

For assembly purposes, the glucosamine residues provide the sites for connection to the chitosan backbone. In their protonated form, the amines allow connection through *electrostatic* interactions.[22] The *nucleophilic* properties of the amines allow connections through covalent linkages that can be formed through a range of coupling chemistries.[23,24] Finally, chitosan's *metal binding* properties[25–28] allow connections through chelation mechanisms.[29,30]

Macromolecular length scale (1–100 nm)

Chitosan is large with a typical molecular weight of 10^5–10^6, a radius of gyration of 10^1–10^2 nm,[31–34] and a contour length approaching microns.[35,36] For comparison, chitosan is about 10-fold larger than typical globular proteins and comparable in size to large unilamellar vesicles. In solution, chitosan behaves as a stiff worm-like chain with persistence lengths reported to be 5–15 nm.[31,33,34,37] Even this measure of chain stiffness is large compared to the diameter of typical globular proteins (≈2–5 nm). Chitosan's size and stiffness are expected to vary with pH, salt concentration and possibly even composition (*i.e.*, GlcNAc content), and studies to resolve



these effects are ongoing in several labs. At low pH (<3.5) electrostatic repulsions expand the chitosan chain and make the backbone more rigid.[38] At intermediate pH (4–6), chitosan's radius of gyration appears to remain relatively constant.[32] Further increases in pH leads to precipitation or aggregation.[32,39]

As noted, GlcN's primary amines provide sites for attachment of nano-scale components to the chitosan backbone, through physical, covalent, and metal-binding mechanisms. Fig. 2 illustrates how we have used chitosan's *nucleophilic* properties to connect nano-scale components to its backbone. The left path shows that hydrophobic moieties can be grafted to the chitosan backbone using common reductive amination reactions with long-chain aldehydes.[35,40–43] At sufficiently low degrees of substitution (typically 2%), the chitosan chains remain acid-soluble, while the hydrophobic moieties can insert into the bilayer of surfactant vesicles and physically connect the vesicles to the backbone.[44–46] The right path in Fig. 2 illustrates the grafting of nano-components to chitosan using either chemical or biochemical methods. Various chemical coupling methods have been used to immobilize proteins onto crosslinked and insoluble chitosan supports,[23] or to graft proteins to uncrosslinked and acid-soluble chitosan chains.[47] An alternative biochemical method for coupling proteins to chitosan employs the enzyme tyrosinase to convert accessible tyrosine residues of proteins into reactive *o*-quinones that can react with chitosan's amine.[48] We are aware that this method has been used to graft gelatin,[49,50] green fluorescent protein,[51–53] and silk fibroin[54–57] to chitosan.

Potentially, conjugates of chitosan and nano-components can retain chitosan's *pH-responsive* behavior. This is illustrated in Fig. 3 which shows the pH-solubility profile for the GFP–chitosan conjugate. At low pH, the conjugate is soluble while an increase in pH near or above chitosan's pK_a results in precipitation of the fluorescent conjugate.[51] Studies with the laccase–chitosan conjugate showed that this precipitation is reversible with retention of biological (*i.e.*, laccase's enzymatic) activity upon multiple precipitation and resolubilization steps.[47]

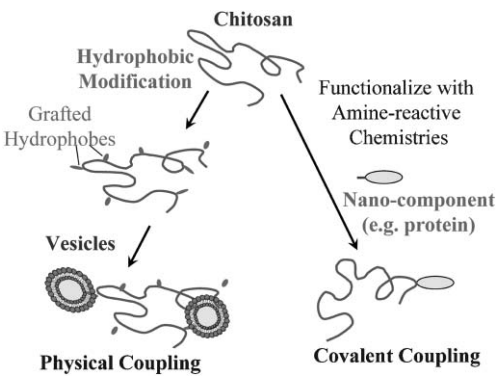


Fig. 2 Two types of mechanisms to couple nano-components to chitosan. The scheme on the left illustrates the *physical coupling* of hydrophobes grafted on chitosan to the bilayers of surfactant vesicles. The scheme on the right depicts the *covalent coupling* of nano-components such as proteins through reactions between their functional groups with the amines on chitosan.

Supramolecular length scale (μm–cm)

As mentioned, chitosan is soluble at low pH but insoluble at neutral or higher pH. While Fig. 3 shows that chitosan can form insoluble precipitates, chitosan can also form a mechanically strong 3-D hydrogel network. For instance, cast chitosan films were observed to have a Young's modulus of 7 MPa when measured under wet conditions.[58] Domard and co-workers proposed three conditions necessary for chitosan to undergo a sol–gel transition to form a homogeneous gel:[59] (i) the chitosan concentration must exceed C^*, the critical concentration for chain overlap ($C^* \approx 0.1\%$[33,60]); (ii) there must be a critical balance between attractive and repulsive interactions; and (iii) the conditions that initiate gel formation must be uniformly percolating. If these conditions are not satisfied, then chitosan chains that undergo the soluble-to-insoluble transition can precipitate as small aggregates without forming a 3-D elastic network. While these conditions provide a useful framework, it is important to recognize that the structural details of the physical crosslinks that serve as the network junctions for the chitosan hydrogels are not well-characterized.[61,62]

Many groups have used chitosan's ability to self-organize in response to a pH-shift to cast/spin films,[28,63–67] spin fibers,[68] or generate 3-D scaffolds by robotic dispensing.[69] Our interest in chitosan has been sparked by the observation that network-formation can be induced by convenient and localized electrical signals as illustrated in Fig. 4.[70,71] Mechanistically, Fig. 4a shows that cathodic reactions that result in the net

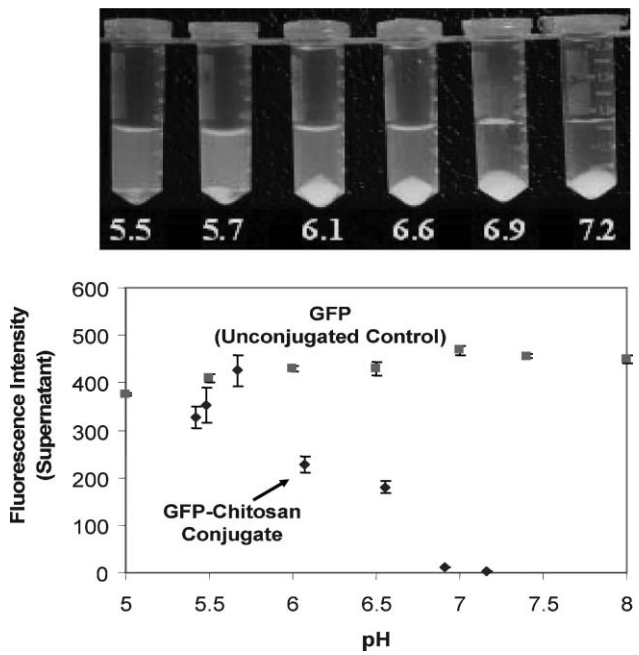


Fig. 3 pH-Responsive properties of GFP–chitosan conjugates. Adapted with permission from ref. 51 (copyright 2003 American Chemical Society). The control solution of unconjungated GFP has a fluorescence that is independent of pH. In contrast, the GFP–chitosan conjugate precipitates when the pH is increased from 5.5 to 7, as shown by the photographs (above), taken under UV illumination. Consequently, the fluorescence in the supernatant solution sharply decreases with pH.

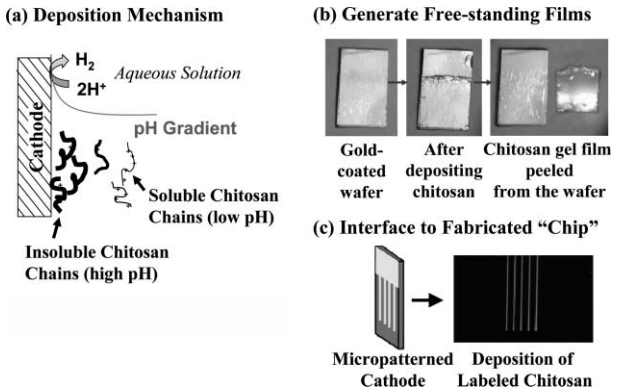


Fig. 4 Chitosan assembly using electrochemical gradients. (A) The local gradient of pH at a cathode results in the deposition of a chitosan network from solution. (B) Electro-deposited chitosan networks can be peeled from gold wafers as "free standing" films. (C) Chitosan can also be deposited onto micropatterned cathodes. The figure on the right shows localized deposition of a fluorescein-labeled chitosan. The stripes on the pattern are 20 μm wide and the separation between the stripes is 100 μm.

consumption of H^+ (or production of OH^-) can generate a localized region of high pH adjacent to the cathode surface. Chitosan chains that experience this localized high pH can be induced to undergo gelation[72] with the formation of thin films or thicker hydrogels depending on the electrodeposition conditions.[71] Thick chitosan films can be peeled from the wafer as illustrated in Fig. 4b and thus electrodeposition can be used to generate "free-standing" films. Alternatively, chitosan can be electrodeposited onto micropatterned electrodes as illustrated in Fig. 4c and this electrodeposition can be performed with micron-level lateral selectivity.[73] It is important to note that electrodeposited films are stable and remain intact in the absence of an applied voltage, but these films can be readily re-solubilized by washing with weak acids. Alternatively, chitosan films can be made acid-insoluble by covalent crosslinking (*e.g.*, with glutaraldehyde).

Chitosan's electodeposition provides a versatile means for assembling nano-scale components into macro-scale structures, and Fig. 5 illustrates three approaches. Fig. 5a shows that discrete nano-scale components can be blended into the deposition solution and co-deposited with chitosan. Upon deposition, these components can be entrapped within the chitosan film's network. As shown in Table 1 co-deposition with chitosan has been used to assemble various nano-scale components onto electrode surfaces. Since electrodeposition is performed under mild aqueous conditions co-deposition can be used to assemble soft nano-components such as proteins and vesicles. Alternatively, Fig. 5b shows that covalent conjugates of chitosan that retain pH-responsive properties can be electrodeposited. To our knowledge, the only covalent conjugates that have been electrodeposited are chitosan conjugates of either the green fluorescent protein (GFP) or gelatin. These protein–chitosan conjugates were electrodeposited onto micropatterned electrodes either individually[51] or sequentially at separate electrode addresses.[74] Finally, Fig. 5c shows that chitosan can be electrodeposited first, and then the nucleophilic amines can be employed for subsequent

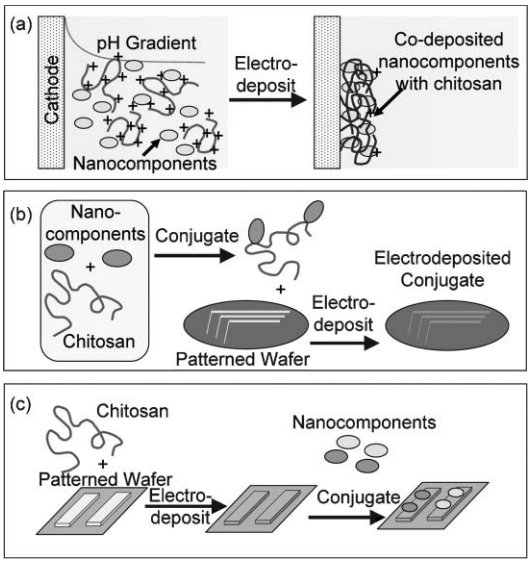


Fig. 5 Three approaches for supramolecular assembly of nano-scale components *via* electrodeposition. (A) Co-deposition of nano-components. (B) Deposition of conjugates of chitosan and nano-scale components. (C) Deposition of chitosan, followed by localized conjugation with nano-scale components.

Table 1 Co-deposition of nano-scale components with chitosan

Soft nano-components	Glucose oxidase[81,82]
	Horseradish peroxidase[83]
	Hemoglobin[84]
	Latex beads[88]
	Vesicles and liposomes[89]
Hard nano-components	Hydroxyapatite[90–92]
	Gold nanoparticles[81,83]
	Carbon nanotubes[82]
	Fe_3O_4 magnetic particles[84]

functionalization. This approach has been used to couple oligonucelotide probes[75] and tobacco mosaic virus (TMV) particles to electrodeposited chitosan.[76]

Conclusions. Limitations and opportunities for a soft interconnect

Chitosan can serve as an interconnect in two ways. First, chitosan can serve as a length-scale interconnect for hierarchical assembly. Nano-scale components can be assembled along chitosan's backbone, while chitosan's self-organization allows assembly to the macro-scale. But chitosan's "softness" makes assembly imprecise when viewed through the lens of conventional rigid microelectronics. The nano-scale components cannot be assembled onto the backbone with nano-scale precision (*i.e.*, at a specific sugar residue) nor is the backbone sufficiently rigid for permanent localization. While chitosan's softness may be ill-suited for fabricating conventional microelectonics, it may be well-suited for fabricating soft medical materials.[77–80] Potentially, tissue engineers could enlist chitosan's fabrication capabilities to construct scaffolds with the interconnected channels needed for cell infiltration, as well as the assembled biological cues required to recruit cells,

facilitate their adhesion, guide their migration, and promote their development. Further, chitosan's pH-responsive gel-formation may enable injectable matrices that can be "programmed" to release controlled dosages of multiple drugs for personalized treatments.

Second, chitosan can serve as an interconnect between nano-scale components and hard electronic devices. Chitosan's ability to recognize localized electrical stimuli and respond by electrodepositing as a stable film provides the means for the signal-guided deposition of nano-components at individual electrode addresses. Especially important is that chitosan electrodeposits from aqueous solution under conditions that are sufficiently mild to accommodate the labile nature of soft nano-components (*e.g.*, proteins, vesicles and virus particles). For example, Chen and co-workers[81–84] are co-depositing soft and hard nano-components (*e.g.*, enzymes and carbon nano-tubes) onto electrodes to allow the coupling of biological recognition with electrochemical sensing (*e.g.*, to detect glucose). This group exploits the fact that the co-deposits are in direct contact with an electrode. An additional example is the work of Ghodssi, Rubloff and co-workers[85,86] who are fabricating microsystems that exploit chitosan's ability to be electrodeposited from solution at electrode addresses within a microfluidic channel. Potentially, the ability to electrodeposit chitosan from closed and fully-packaged microfluidic devices could offer unique opportunities for high-throughput, lab-on-a-chip applications.[87]

References

1 H. Yi, L. Q. Wu, W. E. Bentley, R. Ghodssi, G. W. Rubloff, J. N. Culver and G. F. Payne, Biofabrication with Chitosan, *Biomacromolecules*, 2005, **6**(6), 2881–2894.

2 K. M. Varum, M. W. Anthonsen, H. Grasdalen and O. Smidsrod, High-Field NMR-Spectroscopy of Partially *N*-Deacetylated Chitins (Chitosans). 3. C-13-NMR Studies of the Acetylation Sequences in Partially *N*-Deacetylated Chitins (Chitosans), *Carbohydr. Res.*, 1991, **217**, 19–27.

3 K. M. Varum, M. W. Anthonsen, H. Grasdalen and O. Smidsrod, High-Field NMR-Spectroscopy of Partially *N*-Deacetylated Chitins (Chitosans). 1. Determination of the Degree of *N*-Acetylation and the Distribution of *N*-Acetyl Groups in Partially *N*-Deacetylated Chitins (Chitosans) by High-Field NMR-Spectroscopy, *Carbohydr. Res.*, 1991, **211**(1), 17–23.

4 T. Serizawa, H. Goto, A. Kishida, T. Endo and M. Akashi, Improved alternate deposition of biodegradable naturally occurring polymers onto a quartz crystal microbalance, *J. Polym. Sci., Part A: Polym. Chem.*, 1999, **37**(6), 801–804.

5 T. Serizawa, M. Yamaguchi and M. Akashi, Enzymatic hydrolysis of a layer-by-layer assembly prepared from chitosan and dextran sulfate, *Macromolecules*, 2002, **35**(23), 8656–8658.

6 T. Serizawa, M. Yamaguchi and M. Akashi, Alternating bioactivity of polymeric layer-by-layer assemblies: anticoagulation *vs.* procoagulation of human blood, *Biomacromolecules*, 2002, **3**(4), 724–731.

7 T. Serizawa, M. Yamaguchi, T. Matsuyama and M. Akashi, Alternating bioactivity of polymeric layer-by-layer assemblies: anti- *vs.* procoagulation of human blood on chitosan and dextran sulfate layers, *Biomacromolecules*, 2000, **1**(3), 306–309.

8 O. Etienne, A. Schneider, C. Taddei, L. Richert, P. Schaaf, J. C. Voegel, C. Egles and C. Picart, Degradability of polysaccharides multilayer films in the oral environment: an *in vitro* and *in vivo* study, *Biomacromolecules*, 2005, **6**(2), 726–733.

9 L. Richert, P. Lavalle, E. Payan, X. Z. Shu, G. D. Prestwich, J. F. Stoltz, P. Schaaf, J. C. Voegel and C. Picart, Layer by layer buildup of polysaccharide films: physical chemistry and cellular adhesion aspects, *Langmuir*, 2004, **20**(2), 448–458.

10 B. Thierry, F. M. Winnik, Y. Merhi, J. Silver and M. Tabrizian, Bioactive coatings of endovascular stents based on polyelectrolyte multilayers, *Biomacromolecules*, 2003, **4**(6), 1564–1571.

11 Y. Lvov, M. Onda, K. Ariga and T. Kunitake, Ultrathin films of charged polysaccharides assembled alternately with linear polyions, *J. Biomater. Sci., Polym. Ed.*, 1998, **9**(4), 345–355.

12 H. Z. Huang and X. R. Yang, Chitosan mediated assembly of gold nanoparticles multilayer, *Colloids Surf., A*, 2003, **226**(1–3), 77–86.

13 T. I. Croll, A. J. O'Connor, G. W. Stevens and J. J. Cooper-White, A blank slate? Layer-by-layer deposition of hyaluronic acid and chitosan onto various surfaces, *Biomacromolecules*, 2006, **7**(5), 1610–1622.

14 G. Berth, A. Voigt, H. Dautzenberg, E. Donath and H. Mohwald, Polyelectrolyte complexes and layer-by-layer capsules from chitosan/chitosan sulfate, *Biomacromolecules*, 2002, **3**(3), 579–590.

15 L. Coche-Guerente, J. Desbrieres, J. Fatisson, P. Labbe, M. C. Rodriguez and G. Rivas, Physicochemical characterization of the layer-by-layer self-assembly of polyphenol oxidase and chitosan on a glassy carbon electrode, *Electrochim. Acta*, 2005, **50**(14), 2865–2877.

16 M. W. Anthonsen and O. Smidsrod, Hydrogen-Ion Titration of Chitosans with Varying Degrees of *N*-Acetylation by Monitoring Induced H-1-NMR Chemical-Shifts, *Carbohydr. Polym.*, 1995, **26**(4), 303–305.

17 P. Sorlier, A. Denuziere, C. Viton and A. Domard, Relation between the Degree of Acetylation and the Electrostatic Properties of Chitin and Chitosan, *Biomacromolecules*, 2001, **2**(3), 765–772.

18 P. Sorlier, C. Viton and A. Domard, Relation between solution properties and degree of acetylation of chitosan: role of aging, *Biomacromolecules*, 2002, **3**(6), 1336–1342.

19 M. Rinaudo, G. Pavlov and J. Desbrieres, Influence of acetic acid concentration on the solubilization of chitosan, *Polymer*, 1999, **40**, 7029–7032.

20 K. M. Varum, M. H. Ottoy and O. Smidsrod, Water-solubility of partially *N*-acetylated chitosans as a function of pH: effect of chemical composition and depolymerization, *Carbohydr. Polym.*, 1994, **25**, 65–70.

21 S. P. Strand, K. Tommeraas, K. M. Varum and K. Ostgaard, Electrophoretic light scattering studies of chitosans with different degrees of *N*-acetylation, *Biomacromolecules*, 2001, **2**(4), 1310–1314.

22 L. Gomez, H. L. Ramirez, A. Neira-Carrillo and R. Villalonga, Polyelectrolyte complex formation mediated immobilization of chitosan-invertase neoglycoconjugate on pectin-coated chitin, *Bioprocess Biosyst. Eng.*, 2006, **28**(6), 387–395.

23 B. Krajewska, Application of chitin- and chitosan-based materials for enzyme immobilizations: a review, *Enzyme Microb. Technol.*, 2004, **35**(2–3), 126–139.

24 H. W. Wang, Y. M. Dong and Y. Q. Zhao, Advance of graft copolymerization onto chitin and chitosan, *Huaxue Jinzhan*, 2006, **18**(5), 601–608.

25 E. P. Kuncoro, J. Roussy and E. Guibal, Mercury recovery by polymer-enhanced ultrafiltration: comparison of chitosan and poly(ethylenimine) used as macroligand, *Sep. Sci. Technol.*, 2005, **40**(1–3), 659–684.

26 I. M. N. Vold, K. M. Varum, E. Guibal and O. Smidsrod, Binding of ions to chitosan — selectivity studies, *Carbohydr. Polym.*, 2003, **54**(4), 471–477.

27 C. L. Schauer, M. S. Chen, M. Chatterley, K. Eisemann, E. R. Welsh, R. R. Price, P. E. Schoen and F. S. Ligler, Color changes in chitosan and poly(allyl amine) films upon metal binding, *Thin Solid Films*, 2003, **434**(1–2), 250–257.

28 C. L. Schauer, M. S. Chen, R. R. Price, P. E. Schoen and F. S. Ligler, Colored thin films for specific metal ion detection, *Environ. Sci. Technol.*, 2004, **38**(16), 4409–4413.

29 E. Guibal, Interactions of metal ions with chitosan-based sorbents: a review, *Sep. Purif. Technol.*, 2004, **38**(1), 43–74.

30 R. A. Sufi, A. B. Kelly and T. A. Barbari, Controlling the orientation of immobilized proteins on an affinity membrane through chelation of a histidine tag to a chitosan-Ni++ surface, *J. Membr. Sci.*, 2006, **282**(1-2), 311–321.

31 J. Brugnerotto, J. Desbrieres, G. Roberts and M. Rinaudo, Characterization of chitosan by steric exclusion chromatography, *Polymer*, 2001, **42**(25), 9921–9927.

32 C. Schatz, C. Pichot, T. Delair, C. Viton and A. Domard, Static light scattering studies on chitosan solutions: from macromolecular chains to colloidal dispersions, *Langmuir*, 2003, **19**(23), 9896–9903.
33 C. Schatz, C. Viton, T. Delair, C. Pichot and A. Domard, Typical physicochemical behaviors of chitosan in aqueous solution, *Biomacromolecules*, 2003, **4**(3), 641–648.
34 G. Lamarque, J. M. Lucas, C. Viton and A. Domard, Physicochemical behavior of homogeneous series of acetylated chitosans in aqueous solution: role of various structural parameters, *Biomacromolecules*, 2005, **6**(1), 131–142.
35 C. Esquenet, P. Terech, F. Boue and E. Buhler, Structural and rheological properties of hydrophobically modified polysaccharide associative networks, *Langmuir*, 2004, **20**(9), 3583–3592.
36 G. Berth, H. Colfen and H. Dautzenberg, Physicochemical and chemical characterisation of chitosan in dilute aqueous solution, *Prog. Colloid Polym. Sci.*, 2002, **119**, 50–57.
37 J. Brugnerotto, J. Desbrieres, L. Heux, K. Mazeau and M. Rinaudo, Overview on structural characterization of chitosan molecules in relation with their behavior in solution, *Macromol. Symp.*, 2001, **168**, 1–20.
38 J. H. Pa and T. L. Yu, Light scattering study of chitosan in acetic acid aqueous solutions, *Macromol. Chem. Phys.*, 2001, **202**(7), 985–991.
39 P. Sorlier, C. Rochas, I. Morfin, C. Viton and A. Domard, Light scattering studies of the solution properties of chitosans of varying degrees of acetylation, *Biomacromolecules*, 2003, **4**(4), 1034–1040.
40 C. Esquenet and E. Buhler, Phase behavior of associating polyelectrolyte polysaccharides. 1. Aggregation process in dilute solution, *Macromolecules*, 2001, **34**(15), 5287–5294.
41 J. Desbrieres, C. Martinez and M. Rinaudo, Hydrophobic derivatives of chitosan: characterization and rheological behaviour, *Int. J. Biol. Macromol.*, 1996, **19**(1), 21–28.
42 A. L. Kjoniksen, B. Nystrom, C. Iversen, T. Nakken, O. Palmgren and T. Tande, Viscosity of dilute aqueous solutions of hydrophobically modified chitosan and its unmodified analogue at different conditions of salt and surfactant concentrations, *Langmuir*, 1997, **13**(19), 4948–4952.
43 B. Nystrom, A. L. Kjoniksen and C. Iversen, Characterization of association phenomena in aqueous systems of chitosan of different hydrophobicity, *Adv. Colloid Interface Sci.*, 1999, **79**(2–3), 81–103.
44 J. H. Lee, J. P. Gustin, T. H. Chen, G. F. Payne and S. R. Raghavan, Vesicle-biopolymer gels: networks of surfactant vesicles connected by associating biopolymers, *Langmuir*, 2005, **21**(1), 26–33.
45 J.-H. Lee, V. Agarwal, A. Bose, G. F. Payne and S. R. Raghavan, Transition from Unilamellar to Bilamellar Vesicles Induced by an Amphiphilic Biopolymer, *Phys. Rev. Lett.*, 2006, **96**(4)048102-4.
46 C. Zhu, J. H. Lee, S. R. Raghavan and G. F. Payne, Bioinspired vesicle restraint and mobilization using a biopolymer scaffold, *Langmuir*, 2006, **22**(7), 2951–2955.
47 R. Vazquez-Duhalt, R. Tinoco, P. D'Antonio, L. D. T. Topoleski and G. F. Payne, Enzyme conjugation to the polysaccharide chitosan: smart biocatalysts and biocatalytic hydrogels, *Bioconjugate Chem.*, 2001, **12**(2), 301–306.
48 C. Muzzarelli and R. A. A. Muzzarelli, Reactivity of quinones towards chitosan, *Trends Glycosci. Glycotechnol.*, 2002, **14**, 223–229.
49 T. H. Chen, H. D. Embree, E. M. Brown, M. M. Taylor and G. F. Payne, Enzyme-catalyzed gel formation of gelatin and chitosan: potential for *in situ* applications, *Biomaterials*, 2003, **24**(17), 2831–2841.
50 T. H. Chen, H. D. Embree, L. Q. Wu and G. F. Payne, *In vitro* protein–polysaccharide conjugation: tyrosinase-catalyzed conjugation of gelatin and chitosan, *Biopolymers*, 2002, **64**(6), 292–302.
51 T. Chen, D. A. Small, L.-Q. Wu, G. W. Rubloff, R. Ghodssi, R. Vazquez-Duhalt, W. E. Bentley and G. F. Payne, Nature-Inspired Creation of Protein–Polysaccharide Conjugate and its Subsequent Assembly onto a Patterned Surface, *Langmuir*, 2003, **19**, 9382–9386.
52 A. T. Lewandowski, D. A. Small, T. Chen, G. F. Payne and W. E. Bentley, Tyrosine-based "Activatable Pro-Tag": enzyme-catalyzed protein capture and release, *Biotechnol. Bioeng.*, 2006, **93**(6), 1207–1215.
53 R. Fernandes, H. M. Yi, L. Q. Wu, G. W. Rubloff, R. Ghodssi, W. E. Bentley and G. F. Payne, Thermo-biolithography: a technique for patterning nucleic acids and proteins, *Langmuir*, 2004, **20**(3), 906–913.
54 G. D. Kang, K. H. Lee, C. S. Ki, J. H. Nahm and Y. H. Park, Silk fibroin/chitosan conjugate crosslinked by tyrosinase, *Macromol. Res.*, 2004, **12**(5), 534–539.
55 P. Monti, G. Freddi, S. Sampaio, M. Tsukada and P. Taddei, Structure modifications induced in silk fibroin by enzymatic treatments. a Raman study, *J. Mol. Struct.*, 2005, **744**, 685–690.
56 S. Sampaio, P. Taddei, P. Monti, J. Buchert and G. Freddi, Enzymatic grafting of chitosan onto Bombyx mori silk fibroin: kinetic and IR vibrational studies, *J. Biotechnol.*, 2005, **116**(1), 21–33.
57 G. Freddi, A. Anghileri, S. Sampaio, J. Buchert, P. Monti and P. Taddei, Tyrosinase-catalyzed modification of Bombyx mori silk fibroin: grafting of chitosan under heterogeneous reaction conditions, *J. Biotechnol.*, 2006, **125**(2), 281–294.
58 L. Q. Wu, M. K. McDermott, C. Zhu, R. Ghodssi and G. F. Payne, Mimicking Biological Phenol Reaction Cascades to Confer Mechanical Function, *Adv. Funct. Mater.*, 2006, **16**, 1967–1974.
59 N. Boucard, C. Viton and A. Domard, New aspects of the formation of physical hydrogels of chitosan in a hydroalcoholic medium, *Biomacromolecules*, 2005, **6**(6), 3227–3237.
60 J. Desbrieres, Viscosity of semiflexible chitosan solutions: influence of concentration, temperature, and role of intermolecular interactions, *Biomacromolecules*, 2002, **3**, 342–349.
61 A. P. Zhu, M. B. Chan-Park, S. Dai and L. Li, The aggregation behavior of *O*-carboxymethylchitosan in dilute aqueous solution, *Colloids Surf., B*, 2005, **43**(3–4), 143–149.
62 A. P. Zhu, S. Dai, L. Li and F. Zhao, Salt effects on aggregation of *O*-carboxymethylchitosan in aqueous solution, *Colloids Surf., B*, 2006, **47**(1), 20–28.
63 D. S. dos Santos, P. J. G. Goulet, N. P. W. Pieczonka, O. N. Oliveira and R. F. Aroca, Gold nanoparticle embedded, self-sustained chitosan films as substrates for surface-enhanced Raman scattering, *Langmuir*, 2004, **20**(23), 10273–10277.
64 M. G. Zhang, A. Smith and W. Gorski, Carbon nanotube–chitosan system for electrochemical sensing based on dehydrogenase enzymes, *Anal. Chem.*, 2004, **76**(17), 5045–5050.
65 M. G. Zhang and W. Gorski, Electrochemical sensing platform based on the carbon nanotubes/redox mediators-biopolymer system, *J. Am. Chem. Soc.*, 2005, **127**(7), 2058–2059.
66 T. Baumgart and A. Offenhausser, Polysaccharide-supported planar bilayer lipid model membranes, *Langmuir*, 2003, **19**(5), 1730–1737.
67 L. Q. Wu, R. Ghodssi, Y. A. Elabd and G. F. Payne, Biomimetic Pattern Transfer, *Adv. Funct. Mater.*, 2005, **15**, 189–195.
68 S. H. Lim and S. M. Hudson, Review of chitosan and its derivatives as antimicrobial agents and their uses as textile chemicals, *J. Macromol. Sci., Polym. Rev.*, 2003, **C43**(2), 223–269.
69 T. H. Ang, F. S. A. Sultana, D. W. Hutmacher, Y. S. Wong, J. Y. H. Fuh, X. M. Mo, H. T. Loh, E. Burdet and S. H. Teoh, Fabrication of 3D chitosan–hydroxyapatite scaffolds using a robotic dispensing system, *Mater. Sci. Eng., C*, 2002, **20**(1–2), 35–42.
70 L. Q. Wu, A. P. Gadre, H. M. Yi, M. J. Kastantin, G. W. Rubloff, W. E. Bentley, G. F. Payne and R. Ghodssi, Voltage-dependent assembly of the polysaccharide chitosan onto an electrode surface, *Langmuir*, 2002, **18**(22), 8620–8625.
71 R. Fernandes, L. Q. Wu, T. H. Chen, H. M. Yi, G. W. Rubloff, R. Ghodssi, W. E. Bentley and G. F. Payne, Electrochemically induced deposition of a polysaccharide hydrogel onto a patterned surface, *Langmuir*, 2003, **19**(10), 4058–4062.
72 R. A. Zangmeister, J. J. Park, G. W. Rubloff and M. J. Tarlov, Electrochemical study of chitosan films deposited from solution at reducing potentials, *Electrochim. Acta*, 2006, **51**(25), 5324–5333.
73 L.-Q. Wu, H. Yi, S. Li, G. W. Rubloff, W. E. Bentley, R. Ghodssi and G. F. Payne, Spatially-selective Deposition of a Reactive Polysaccharide Layer onto a Patterned Template, *Langmuir*, 2003, **19**, 519–524.
74 H. Yi, L. Q. Wu, R. Ghodssi, G. W. Rubloff, G. F. Payne and W. E. Bentley, Signal-directed sequential assembly of biomolecules on patterned surfaces, *Langmuir*, 2005, **21**(6), 2104–2107.
75 H. M. Yi, L. Q. Wu, R. Ghodssi, G. W. Rubloff, G. F. Payne and W. E. Bentley, A robust technique for assembly of nucleic acid

hybridization chips based on electrochemically templated chitosan, *Anal. Chem.*, 2004, **76**(2), 365–372.
76 H. Yi, S. Nisar, S.-Y. Lee, M. A. Powers, W. E. Bentley, G. F. Payne, R. Ghodssi, G. W. Rubloff, M. T. Harris and J. N. Culver, Patterned Assembly of Genetically Modified Viral Nanotemplates *via* Nucleic Acid Hybridization, *Nano Lett.*, 2005, **5**(10), 1931–1936.
77 M. N. Kumar, R. A. Muzzarelli, C. Muzzarelli, H. Sashiwa and A. J. Domb, Chitosan chemistry and pharmaceutical perspectives, *Chem. Rev.*, 2004, **104**(12), 6017–6084.
78 M. N. V. R. Kumar, A review of chitin and chitosan applications, *React. Funct. Polym.*, 2000, **46**(1), 1–27.
79 J. Berger, M. Reist, J. M. Mayer, O. Felt, N. A. Peppas and R. Gurny, Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications, *Eur. J. Pharm. Biopharm.*, 2004, **57**(1), 19–34.
80 H. Zhang, S. Mardyani, W. C. W. Chan and E. Kumacheva, Design of biocompatible chitosan microgels for targeted pH-mediated intracellular release of cancer therapeutics, *Biomacromolecules*, 2006, **7**(5), 1568–1572.
81 X. L. Luo, J. J. Xu, Y. Du and H. Y. Chen, A glucose biosensor based on chitosan–glucose oxidase–gold nanoparticles biocomposite formed by one-step electrodeposition, *Anal. Biochem.*, 2004, **334**(2), 284–289.
82 X. L. Luo, J. J. Xu, J. L. Wang and H. Y. Chen, Electrochemically deposited nanocomposite of chitosan and carbon nanotubes for biosensor application, *Chem. Commun.*, 2005(16), 2169–2171.
83 X. L. Luo, J. J. Xu, Q. Zhang, G. J. Yang and H. Y. Chen, Electrochemically deposited chitosan hydrogel for horseradish peroxidase immobilization through gold nanoparticles self-assembly, *Biosens. Bioelectron.*, 2005, **21**, 190–196.
84 G. Zhao, J. J. Xu and H. Y. Chen, Fabrication, characterization of Fe_3O_4 multilayer film and its application in promoting direct electron transfer of hemoglobin, *Electrochem. Commun.*, 2006, **8**(1), 148–154.
85 M. J. Kastantin, S. Li, A. P. Gadre, L. Q. Wu, W. E. Bentley, G. F. Payne, G. W. Rubloff and R. Ghodssi, Integrated fabrication of polymeric devices for biological applications, *Sens. Mater.*, 2003, **15**(6), 295–311.
86 M. A. Powers, S. T. Koev, A. Schleunitz, H. Yi, V. Hodzic, W. E. Bentley, G. F. Payne, G. W. Rubloff and R. Ghodssi, A fabrication platform for electrically mediated optically active biofunctionalized sites in BioMEMS, *Lab Chip*, 2005, **5**, 583–586.
87 J. J. Park, X. Luo, H. Yi, T. M. Valentine, G. F. Payne, W. E. Bentley, R. Ghodssi and G. W. Rubloff, Chitosan-mediated *in situ* biomolecule assembly in completely packaged microfluidic devices, *Lab Chip*, 2006, **6**, 1315–1321.
88 L. Q. Wu, K. Lee, X. Wang, D. S. English, W. Losert and G. F. Payne, Chitosan-Mediated and Spatially Selective Electrodeposition of Nanoscale Particles, *Langmuir*, 2005, **21**(8), 3641–3646.
89 C. Zhu, L. Q. Wu, X. Wang, J. H. Lee, D. S. English, R. Ghodssi, S. R. Raghavan and G. F. Payne, Reversible Vesicle Restraint in Response to Spatiotemporally-controlled Electrical Signals: A Bridge Between Electrical and Chemical Signaling Modes, *Langmuir*, 2007, **23**(1), 286–291.
90 J. Redepenning, G. Venkataraman, J. Chen and N. Stafford, Electrochemical preparation of chitosan/hydroxyapatite composite coatings on titanium substrates, *J. Biomed. Mater. Res., Part A*, 2003, **66**(2), 411–416.
91 X. Pang and I. Zhitomirsky, Electrodeposition of composite hydroxyapatite–chitosan films, *Mater. Chem. Phys.*, 2005, **94**, 245–251.
92 X. Pang and I. Zhitomirsky, Electrodeposition of nanocomposite organic–inorganic coatings for biomedical applications, *Int. J. Nanosci.*, 2005, **4**(3), 409–418.