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Glycomics: new challenges and opportunities in regenerative medicine

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Abstract:	Tissue engineering relyes on the possibility to engineer cell microenvironment by means of bioactive materials, biochemical and physical stimuli in order to guide cell behaviour and to regenerate a damaged tissue. Despite the relevance of glycan epitopes as signalling molecules, and the recent advances in glycomics, their use as biomolecular cues at the interface between materials and cells for the controlled stimulation of adhesion and differentiation processes for regenerative medicine applications is still limited. In this concept article we will briefly outline the basis, the impact on health and economics of regenerative medicine, together with the recent applications of the glycocode in tissue regeneration approaches.
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Glycomics: new challenges and opportunities in regenerative medicine

Laura Russo,[a] and Laura Cipolla*[a]

Abstract: Tissue engineering relyes on the possibility to engineer cell microenvironment by means of bioactive materials, biochemical and physical stimuli in order to guide cell behaviour and to regenerate a damaged tissue. Despite the relevance of glycan epitopes as signalling molecules, and the recent advances in glycomics, their use as biomolecular cues at the interface between materials and cells for the controlled stimulation of adhesion and differentiation processes for regenerative medicine applications is still limited. In this concept article we will briefly outline the basis, the impact on health and economics of regenerative medicine, together with the recent applications of the glycocode in tissue regeneration approaches.

Biomaterials and regenerative medicine: a brief introduction

Tissue formation, function and regeneration after damage is the result of a balanced interaction of numerous individual cell fate processes, each of which is induced by an array of signals originating from the extracellular microenvironment.

In case injuries are beyond the natural regeneration limit of the human body, therapeutic treatment is needed. In this respect, medicine is developing innovative approaches and clinical applications referred to as “regenerative medicine”. Regenerative medicine is an extremely interdisciplinary field focused on “the repair, replacement or regeneration of cells, tissues or organs to restore impaired function resulting from any cause, including congenital defects, disease, trauma and aging. It uses a combination of several technological approaches that moves it beyond traditional transplantation and replacement therapies.”[1] Regenerative medicine approaches usually combine key essential components: signalling (macro)molecules (i.e. paracrine factors that include growth factors, cytokines, interleukines, etc.), cells,, and biomaterials. Different combinations of these key elements may be used, affording different therapeutic strategies. However, regardless of the strategy, biomaterial scaffolds quite often are the basis to steer and support cells towards regeneration. The concept of biomaterial evolved in the last century.[2] Initially biomaterials were defined as materials of different chemical nature used as medical implants; they have been used since ancient times, for several thousands years, as witnessed by an egyptian wooden toe prosthesis dating back to 1065–740 bc. The modern era of biomaterials is a consequence of the observation of a British physician[3] in the late 40’; he observed that pilots who had fragments of poly(methyl methacrylate), (PMMA) canopy in their eyes, did not exhibit any adverse biochemical response: PMMA was biocompatible. He deduced that PMMA could be suitable for implant lenses for treating cataracts. Since his first implant (1949) more than five millions patients had intraocular lenses implanted for the treatment of this pathology. In the same period several other bioinert materials were developed, such as cage heart valves, vascular grafts, hip implants. At that time, the biomaterials had to be biocompatible and performed mainly mechanical functions. Since the ‘70s, the advances in molecular biology, genomics, proteomics, biotechnology, material chemistry, engineering and medicine, revolutionised the concept of biomaterials: the biomaterial is not any more bioinert, but it is a bioactive scaffold where signalling biomolecules have been incorporated in order to drive the controlled stimulation of selected target cells through the systematic combination of molecular and physical signals. Nowadays, biomaterials are defined as “nonviable materials intended to interact with biological systems”[2]. They should be able to support tissue regeneration through their interactions with sorrounding cells and/or throught the delivery of signalling cues.

In the last decades the research field and the market of regenerative medicine and biomaterials is experiencing an unprecedented growth, due to increased fundings by government bodies worldwide, (bio)technological advancements, and high demand as a consequence of the increased geriatric population, coupled with growing incidence of cardiovascular diseases, and osteoarthritis. The biomaterial market is expected to reach USD 130.57 billion by 2020, growing at a CAGR of 16% during the forecast period of 2015 to 2020.[4]

Biomaterials are thus considered as instructive environments for the surrounding cells,[5] and an emerging approach in regenerative medicine is to design biomaterials able to establish key and controlled interactions with cells in ways that induce the body’s innate powers of self-repair. In nature, cells gain a variety of information both from surrounding cells and from their microenvironments, that is the extracellular matrix (ECM). The extracellular microenvironment, which surrounds each cells has several important effectors: (i) insoluble matrix molecules (collagen, laminin, elastin or fibronectin), (ii) soluble macromolecules (growth factors, chemokines and cytokines) and (iii) proteins on the surface of neighboring cells. Thus the ultimate fate of a cell toward proliferation, differentiation, migration, and, apoptosis or other specific functions is a coordinated response to the complex molecular interactions with these effectors.

The ECM microenvironment may be mimicked by extrapolation of key properties into biomaterial design.[6] Several material

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properties may be be tuned in order to influence cell behaviour; they include different physical parameters, such as morphology (i.e. fibers, sponges), roughness, topology and topography (at the micro and nano scale),[7] mechanical properties (i.e. stiffness). Significant improvements have been made in recent years in the understanding how the biomaterial physical properties affect biological responses.[8,9] An explanation of these effects is that mechanosensing is an active cellular process involving dynamic interplay between cells and their physical environment.[10] Indeed, several studies demonstrate that physical signals potently guide cell fate and functions.[11]

Surface chemistry (i.e. exposed functional groups) is an additional relevant factor in the interaction with cells, since it influence wettability, protein interactions, and cell behaviour. For example, self-assembled monolayers exposing to cells different chemical functional groups, such as amines, carboxyl or hydroxyl groups have been used to determine how different cell lines interact with differently functionalised surfaces, modulating adhesion, proliferation and differentiation.[12] It was recently demonstrated that small functional groups tethered to material surfaces have a direct influence on the differentiation fate of hMSCs: for example phosphate groups lead to osteogenesis, while hydrophobic *t*-butyl groups induce adipogenesis.[13]

Moreover, the biomaterial can be further decorated with signalling cues in order to specifically drive cell response. Since nature uses definite functional and structural proteins to drive cell behaviour, several efforts have been focused on the "biodecoration" of material surfaces with selected signaling cues derived from proteins. Among them, growth factors[14] small adhesive peptidic sequences[14b, 15] derived from laminin, such as RGD,[16] YIGSR, LGTIPG, IKVAV, PDGSR, LRE, LRGDN and IKLLI,[17] or from type I collagen and fibronectin, i.e. DGEA, KQAGDV, REDV and PHSRN, short signalling peptides,[18] and small molecules[19] have been used for material bioactivation.

It is well known that glycans bring a wealth of information, that is frequently referred to as the glycocode. They hence appear as envaluable biomolecular cues for the decoration of material surfaces in order to modulate cell-material interactions and to guide cell behaviour. To date, the majority of glycan-related studies in the regenerative medicine field has relied only on those available in bulk quantities such as complex polysaccharides, such as proteoglycans chondroitin sulphate,[20] hyaluronic acid,[21] chitosan, and alginates.[22] However, the surface modification of biomaterials with low molecular weight carbohydrate epitopes has been underexploited, and systematic studies on how small carbohydrate epitopes might influence cell adhesion and differentiation processes are now strongly emerging.

In this Concept article we will focus on the outgrowth of glycochemistry in biomaterial decoration, and on the potentialities of glycomics in regenerative medicine applications.

Glycochemistry at the interface

Naturally derived ECM has proved effective in many basic and clinical applications. However, the need for synthetic biomaterials for tissue-specific biological investigations is necessary to gain more control over the cellular behavior. The ECM microenvironment is characterized by a great variety of glycidic based polisaccharides and motifs, ranging from glycosaminoglycans (GAGs) and proteoglycans (PGs), to glycoproteins and glycolipids able to interact whit cell surface receptors. These glycosignatures are able to drive several cellular processes in healthy and pathological states.. The glycocode is decifered by a number of endogenous glycan-binding proteins (GBPs), that include cell-adhesion molecules (i.e. lectins, integrins, cadherins, and selectins) and glycosamynoglycans-binding proteins. Since glycans are involved in many biological processes, it is evident that molecular defects in glycan synthesis and structure are related to an increasing number of pathologies. Many glycan structural variants are now considered as biomarkers and represent therapeutic as well as diagnostic targets.[23]

Overall, cells are immersed in a higly glycosylated microenvironment or "niche" that codes for a variety of functions such as immune responce, cell adhesion, traficking and differentiation. As a consequence, different combination, and spatial organization of small glycan structures and materials may give rise to different types of biomaterial scaffolds that will interact specifically with any given cell line..

Thus, glycoengneering the cell microenvironment by the design of artificial or natural-derived biomaterial scaffolds may open new avenues in regenerative medicine.

Glycoengineered materials: synthetic strategies

Despite carbohydrates might be entrapped in biomaterials and ECM mimics by non-covalent approaches, the most interesting strategy is their covalent immobilisation on the material of choice, since in this way thay cannot diffuse and they can be more resistant to enzymatic degradation. In this case, particular attention should be given on the immobilisation strategy, that should guarantee the control over the spatial orientation of the glycan on the material, in order to ensure the recognition process by the complementary cell receptors.

In order to obtain glycoengineered materials, two main strategy may be used for the immobilisation of the glycan moiety i) the conjugation step is performed on suitable building blocks or monomers, before material fabrication (Fig. 1A) or ii) directly on the bulk material, dwonstream to its preparation (Fig. 1B).

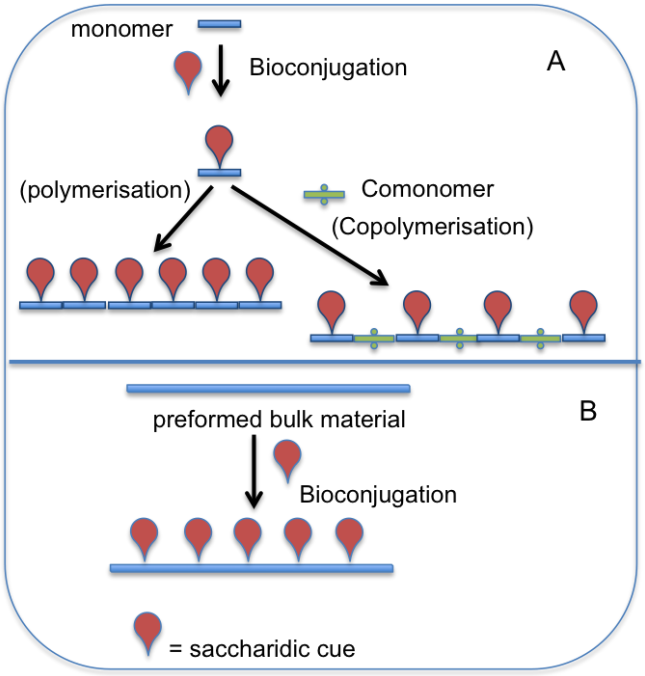

Figure 1. Strategies towards glycoengineered materials. A. A suitable monomer is bioconjugated to the saccharidic cues, then polymerised (left) or copolymerised (right) to afford the "glyco" material; B. The saccharidic cues are bioconjugated to the bulk material by suitable chiemistry.

Regardless of the strategy, both the glycan and the building block/material must be equipped with suitable and mutually reacting functional groups (Figure 2). In this respect, two main key issues should be considered: i) the availability of suitably functionalised synthetic glycan structures and ii) suitable chemical strategies for bioconjugation. Chemoslective bioconjugation reactions have been applied to different research fields,[24] such as glycoconjugate synthesis,[25] glycoarrays,[26] metabolic cell glycoengineering,[27] and only more recently to materials for biomedical applications.[28] The main strategies are reported in Figure 2 and reviewed elsewhere.[29]

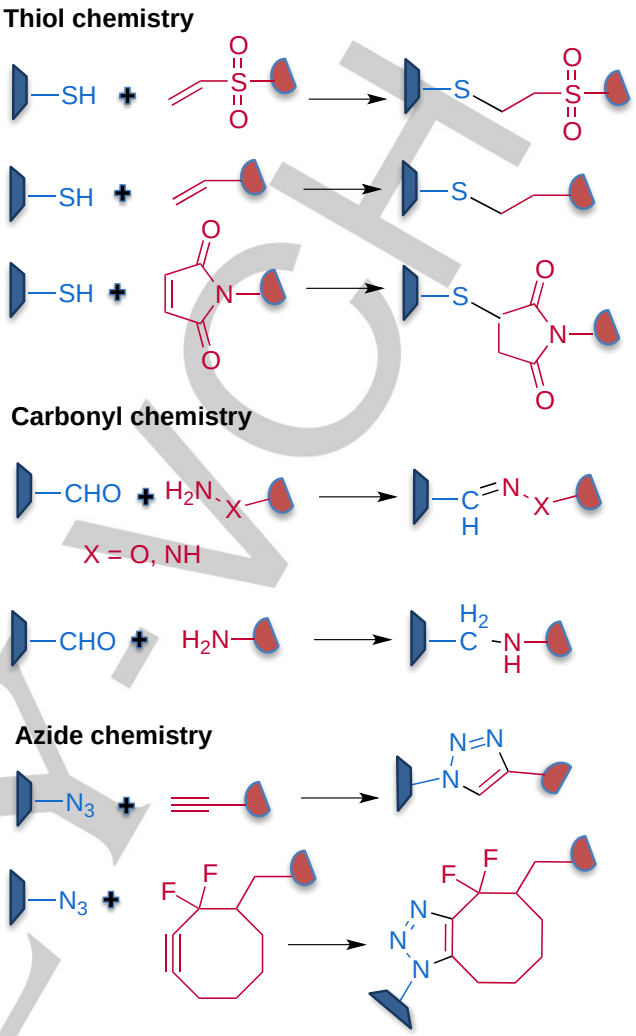

Figure 2. Selected chemoselective strategies for bioconjugation.

Glycoengineered materials: applications for regenerative medicine strategies

Numerous materials have been investigated to obtain artificial ECM. Natural biopolymers, such as collagen, hyaluronic acid, fibronectin, chitosan, alginate, and silk, could be expected to be well accepted as scaffolds in physiological environments.[30]
Mono-, di- and trisaccharides have been employed as simple carbohydrate cues and conjugated to several materials. In many case, the underlying mechanism mediated by glycans is not clear yet and great efforts are still needed for their elucidation. Care must be taken in interpreting results. Promising glycans for tissue regeneration must be experimentally validated for every cell type.
In order to obtain a promising biomaterial for regenerative medicine, a relevant issue is its ability to support cell adhesion and spreading. For example, to improve the biocompatibility of implanted prostheses it may be relevant the development of

scaffolds promoting recolonization by primary cells. Cell adhesion properties of a given material may vary upon cell lineage. While the use of specific adhesive peptidic sequences, such as the RGD motif, has been extensively investigated, systematic studies on adhesive properties of carbohydrates are still scarse.[31]

Galactose, grafted in different forms to several materials (Figure 3), is the first[32] and most investigated carbohydrate for its adhesive properties. Galactose moieties were grafted to different natural (alginate, scaffold **7**, Fig. 3) or artificial (polystyrene, scaffolds **1-4**, PLGA, scaffold **5**, and polyacrilic acid, scaffold **6**) polymeric materials through suitable linkers (scaffolds **3**, **4**, and **6**, Fig. 3), or exposed as the non-reducing unit of lactose derivatives (scaffolds **1**, **2**, **5**, and **7**, Fig. 3). Thus, detailed studies regarding the nature of the bound ligand exposing galactose residues, its orientation and density have been conducted.[33] Galactose supports hepatocytes adhesion and enhances cell functions such as albumin secretion and urea synthesis as a consequence of the interaction with the asialoglycoprotein receptor (ASGPR).[33b] The stereochemistry of the glycosidic bond of galactosides (scaffolds **1** and **2**, Fig. 3) is a relevant feature for the interaction with ASGPR; it was shown that hepatocytes adhesion and spreading was better sustained by β-galactosides, if compared to α-galactosides. In addition, it was demonstrated that galactose grafted to material surfaces provides also selectivity aginst different cell lines: preferential adhesion of hepatocytes was observed in co-culture with fibroblasts, the attachment of which was essentially inhibited.[34]

FIGURE 3 IS AT THE END OF THE MANUSCRIPT (IT IS IN A DOUBLE COLUMN FORMAT)

Figure 3. Different "galactosylated" materials used for the detailed studies of hepatocytes behaviour.

Besides the detailed studies about galactose derivatives in promoting adhesion of hepatocytes, other saccharides were studied with different cell lines. In general, the possibility to induce differential adhesion of cells is gaining much interest for co-culture strategies in order to develop biomimetic structures for tissue engineering.[35] In this respect, a few studies show that small glycan structure (i.e. mono- or disaccharides) may have a role. For example, lactose and mannose were grafted to poly(L-glutamic) acid (PGA, scaffolds **8** and **9**, Fig. 4) and used in multilayer films in combination with poly(L-lysine) in order to investigate their role on the adhesion of chondrocytes as primary cells as compared to that of a tumor cell line, as chondrosarcoma.[36] While lactose only slightly affected adhesion of both cell lines, mannose grafted films sustained adhesion and proliferation of chondrocytes, while chondrosarcoma cells did not grew efficiently. The authors speculate of a possible presence of mannose receptors on chondrocytes, as also reported by Howard ad coworkers.[37] The type-1 glucose transporter (GLUT-1) was found to be implicated in mediating the adhesion of erythrocytes,[38] and chondrocytes.[39] The role of glucose in erythrocytes adhesion was evaluated with different glucose-grafted polystyrene materials (scaffold **1**, Fig. 3 and scaffolds **10**, **11**, Fig. 4). It was demonstrated that a proper interaction with GLUT1 could occur only with glucose grafted to polystyrene through the 3-OH, thus being presented to the receptor in its reducing form (scaffold **10**, Fig. 4); this evidence highlights the extreme relevance of the control of the spatial orientation of carbohydrate cues on material surfaces.

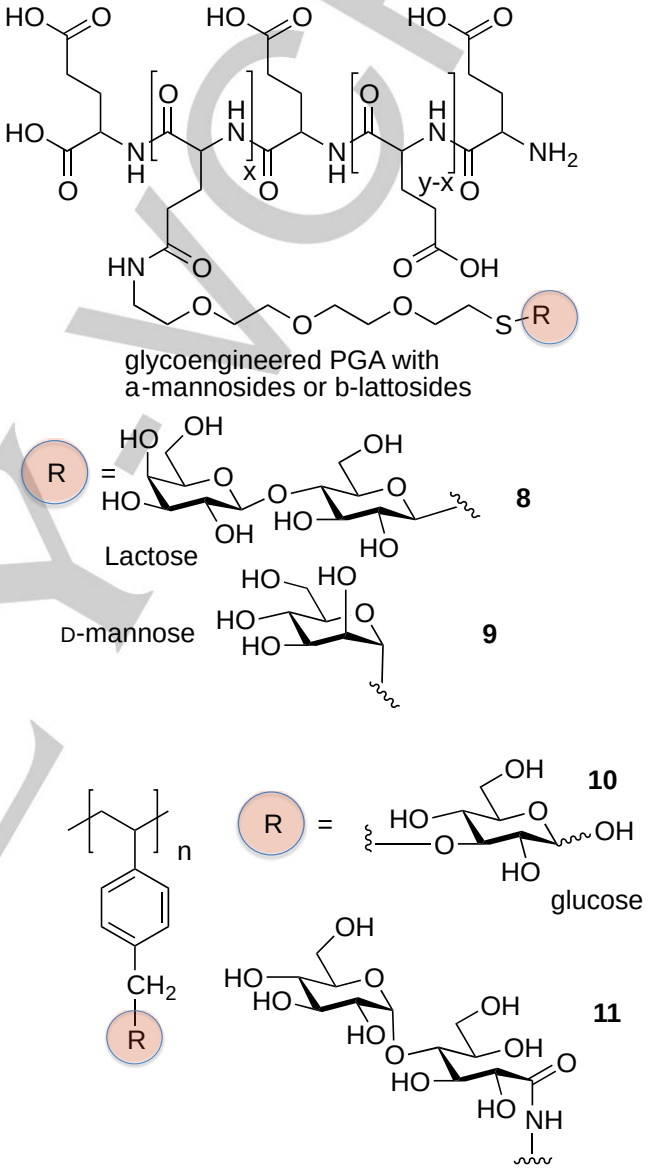

Figure 4. PGA and polystyrene glycoengineered scaffolds used for differential cell culture studies.

Chondrocyte adhesion was evaluated with D- and L-glucose grafted on glass surfaces. The experiments showed that D-glucose surfaces provided a suitable cell microenvironment able to maintain the chondrocytic phenotype.

Several di- and trisaccharides, namely lactose, *N*-acetyl-chitobiose, cellobiose, cellotriose, mannotriose and maltotriose, were grafted *via* carbonyl-alkoxyamine chemoselective

bioconjugation to PMMA coatings and their interaction with human fibroblast was investigated.[40] The study highlighted that phytoglycans such as cellobiose and cellotriose improved fibroblast adhesion if compared to the non-coated surface or to the other saccharide-coated PMMA. On the contrary, when cellobiose and cellotriose were added in soluble form to the culture medium, fibrobalst adhesion to polystyrene plates was inhibited in a concentration-dependent manner. It should be noted that cellobiose and cellotriose are not belonging to the human glycocode.

Among synthetic polymers, aliphatic polyesters such as poly(ε-caprolactone) (PCL) are promising materials for tissue regeneration, due to a unique combination of biodegradability and biocompatibility properties. However, as any other synthetic polymer, it does not present molecular motifs for cell adhesion. In order to improve adhesive properties, PCL-based scaffolds were grafted with N-acetyl-α-D-glucosamine and non-reducing galactose units. In both cases, cell viability over time,, adhesion and spreading of human mesenchymal stem cell (hMSC) was improved if compared to the unfunctionalised scaffolds.[41]

The functionalisation with small carbohydrate epitopes of inert materials such as metals, metal alloys and ceramics may improve their biocompatibility and adhesive properties. For example, stainless steel that can be used for medical implants was functionalised with *N*-acetyl-α-D-glucosamine or D-galactose, *via* a suitable glycoalkyl trimethoxysilane after the activation of the metal surface by silanization (scaffolds **12** and **13**, Fig. 5).[42] The functionalisation of the organosilane was performed by a thiol-ene click reaction between the octenyl glycoside and the trimethoxymercaptopropyl silane. On the other hand, bioceramics are useful biomimetic composite materials for bone tissue regeneration. However, ceramics usually do not possess bioactive properties, and the immobilization of bioactive molecules is an interesting strategy to improve their biological interactions with cells. Toward this aim, α-D-glucosides were conjugated to nanostructured carbonated hydroxyapatite, possessing chemical similarity to the inorganic phases of bones. The propargyl α-D-glucoside was "clicked" to azidated hydroxyapatite (scaffold **14**, Fig. 5).[43]

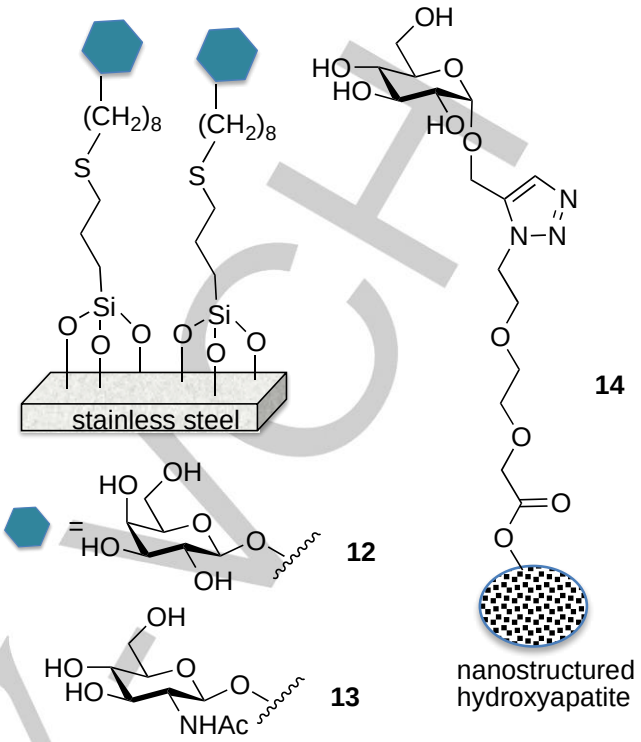

Figure 5. Glycoengineered stainless steel and hydroxyapatite materials.

A further upgrade of scaffolds for regenerative medicine is the use of molecular signals driving stem cell to differentiation. In this respect, only very few examples that exploit the use of small glycan epitopes as differentiating cues do exist..

Stem cell-derived hepatocyte provides the forefront for clinical applications for liver regeneration therapies. Poly(N-p-vinylbenzyl-4-O-β-D-galactopyranosyl-(1→4)-D-gluconamide, scaffold **1**, Fig. 3) displaying non-reducing galactose residues on the polymer surface, together with E-cadherin was used to promote and sustain differentiation of monolayer cultured mouse embrionic stem-cell (mESC) to functional hepatocytes. It has been postulated that the grafted galactose residues induce early expression of ASGPR, sustaining differentiation.[44] In addition, the carbohydrate moieties also contributed to induce cell aggregations and to maintain hepatocytes functionality. RT-PCR experiments performed for albumin (ALB), hepatocyte nuclear factor 4α (HNF-4α), ASGPR, tryptophan oxygenase (mTO) and glucose 6-phosphate (G6P) showed that the hepatocytes cultured on galactose-containing layers expressed high level of liver specific genes compared to the cells grown on control material lacking the carbohydrate. From the numerous studies on galactose-functionalised materials, it appears evident that the design of galactose-grafted biomaterials may contribute to liver tissue engineering.

Collagen 2D films decorated with different non-reducing monosaccharides (galactose, glucose) and sialic acid containing disaccharides were studied for their adhesive and differentiating properties on different cell lines. Neoglycosylation of collagen by reductive amination between amino groups of amino acid side

chains (lysine and hydroxylysine) and the reducing end of the saccharides afforded scaffolds **15**-**17** (Figure 6).

Glycans have been shown to have pivotal roles in nervous system development, regeneration and synaptic plasticity. The glycoengineering of materials with functionally active glycans might add another dimension to neural tissue regeneration. As proof of concept, neuroblastoma F11 cell behaviour was investigated with scaffold **15**.[45] Morphological and functional analysis revealed neuritic-like processes, the presence of the late differention neuronal marker β-tubulinIII and characteristic neuronal electrical activity when cells were cultured on neoglucosylated collagen.. Both morphological and functional analysis showed that neoglucosylated collagen films were able to drive cells to differentiation into active neurons without the use of classical differentiating agents (i.e. retinoic acid). The same chemical procedure with 3'-sialyllactose and 6'-sialyllactose afforded scaffolds **16** and **17** (Fig. 6). Preliminary *in vitro* study on the behavior of mesenchymal stem cells (MSCs)[46] in terms of cell viability, proliferation and induction of osteogenic and chondrogenic related genes has been performed. Results indicate that sialoside epitopes on collagen surface represent a suitable support for MSCs adhesion and cell proliferation; moreover, the neoglycosylation provides MSCs with different and specific stimuli, saccharide-type dependent, in terms of expression of osteogenic and chondrogenic related genes. In particular, the 3'-sialoside (scaffold **17**) significantly upregulates the expression of RUNX2 and ALP, well-known markers of osteogenesis, whereas the 6'-sialoside (scaffold **16**) up-regulates the expression of chondrocyte marker ACAN. Because no osteogenic or chondrogenic supplements in culture media were added, the inductive effect in terms of increased gene expression has to be ascribed uniquely to the presence of carbohydrates onto collagen surface. These results support the promising role of sialosides in the regulation of stem cells fate and open brilliant perspective toward osteochondral tissue engineering applications.

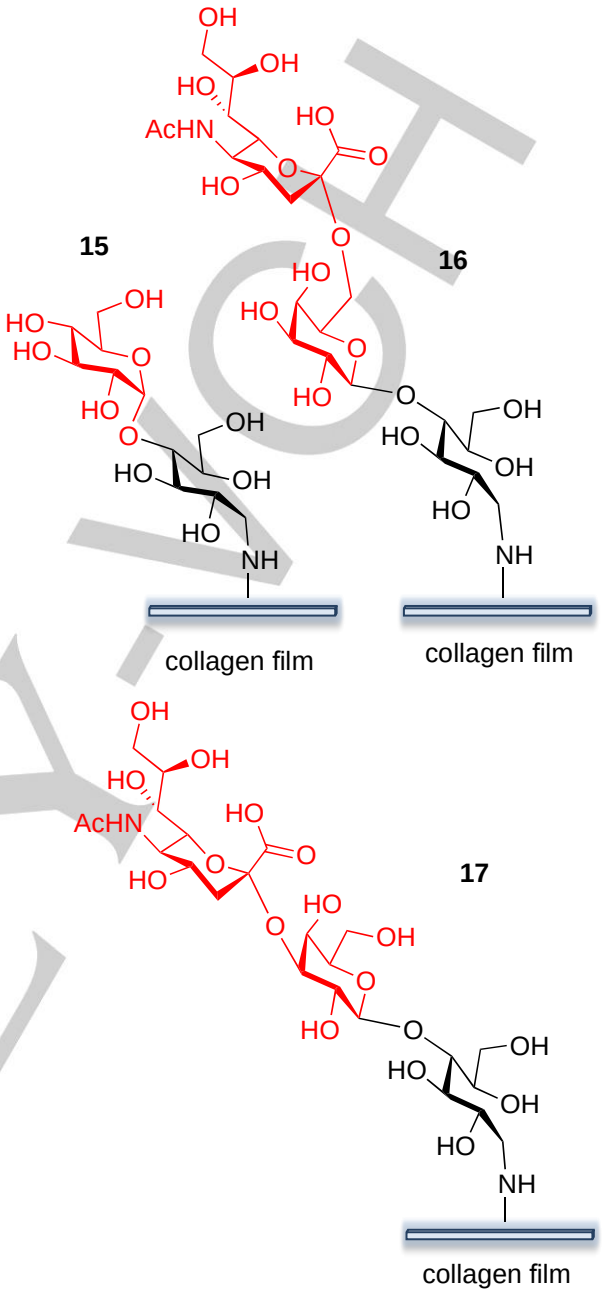

Figure 6. Neoglycosylated collagen scaffolds by reductive amination.

Another example of collagen glycoengineering is given by a thiol-ene reaction between allyl glycosides and the thiolated material surface affording scaffolds **18** and **19** (Fig. 7).[47]

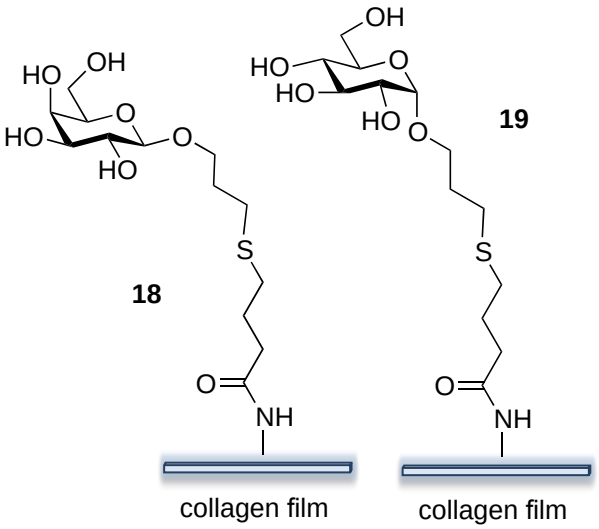

Figure 7. Neoglycosylated collagen films by thiol-ene click chemistry.

Preliminary biological assays were performed implanting unglycosylated and glycosylated collagen films in osteoarthritic animal models. Loss of spontaneous mobility is usually a consequence of joint osteoarthritis, due to cartilage damages. The loss of motility and the recovery related to the tissue damage can be evaluated by the analysis of animal walking pattern by recording its footprints and calculating the sciatic function index (Walking Track Analysis, WTA). The WTA analysis evidenced that neoglycosylated collagen was more effective in promoting motor functional recovery than the collagen itself. These results indicate that small carbohydrate epitopes might influence cartilage repair.

Conclusions and outlook

It is clear that glycans and human diseases are strongly interconnected, including chronic as well as acute syndromes. Detailed studies on the role of the glycocode in healthy and pathological states demonstrate that glycans in their uncorrect or disregulated glycoforms are involved in several high impact diseases, like neurological disorders and mysfunctions, aging-associated diseases, tumours, and inflammation. Morever, glycans are active determinants of stem cell fate. The ability to mimic glycan roles in a tissue-specific manner will afford new opportunities to unravel the complexity of glycan-mediated processes, and might open new avenues in regenerative medicine strategies. Recent advances in tissue engineering and regenerative medicine will probably rely on the future development of glycan engineering technologies and functional glycomics. The attachment of glycans to various biomaterials could help mimic the natural presentation of sugars as glycoproteins and dissect the importance of different protein-carbohydrate combinations. The development of suitable systems for regenerative medicine will require continued crossing of disciplines, that heavily includes chemistry, but also the complementary contribution of medicine, biotechnology and biology, engineering and material science.
Finally, the development of glycoengineered materials is not possible without the establishment of robust glycochemistry, that allows the assembly of a sufficient bulk of diverse carbohydrate motifs suitable for material functionalisation.

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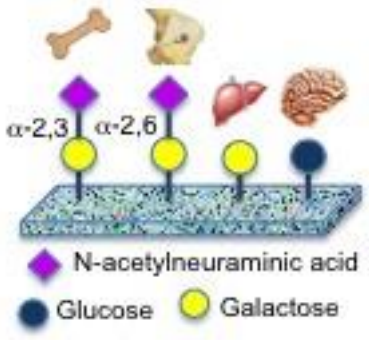

*Laura Russo, Laura Cipolla**

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Glycomics: new challenges and opportunities in regenerative medicine

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CONCEPT

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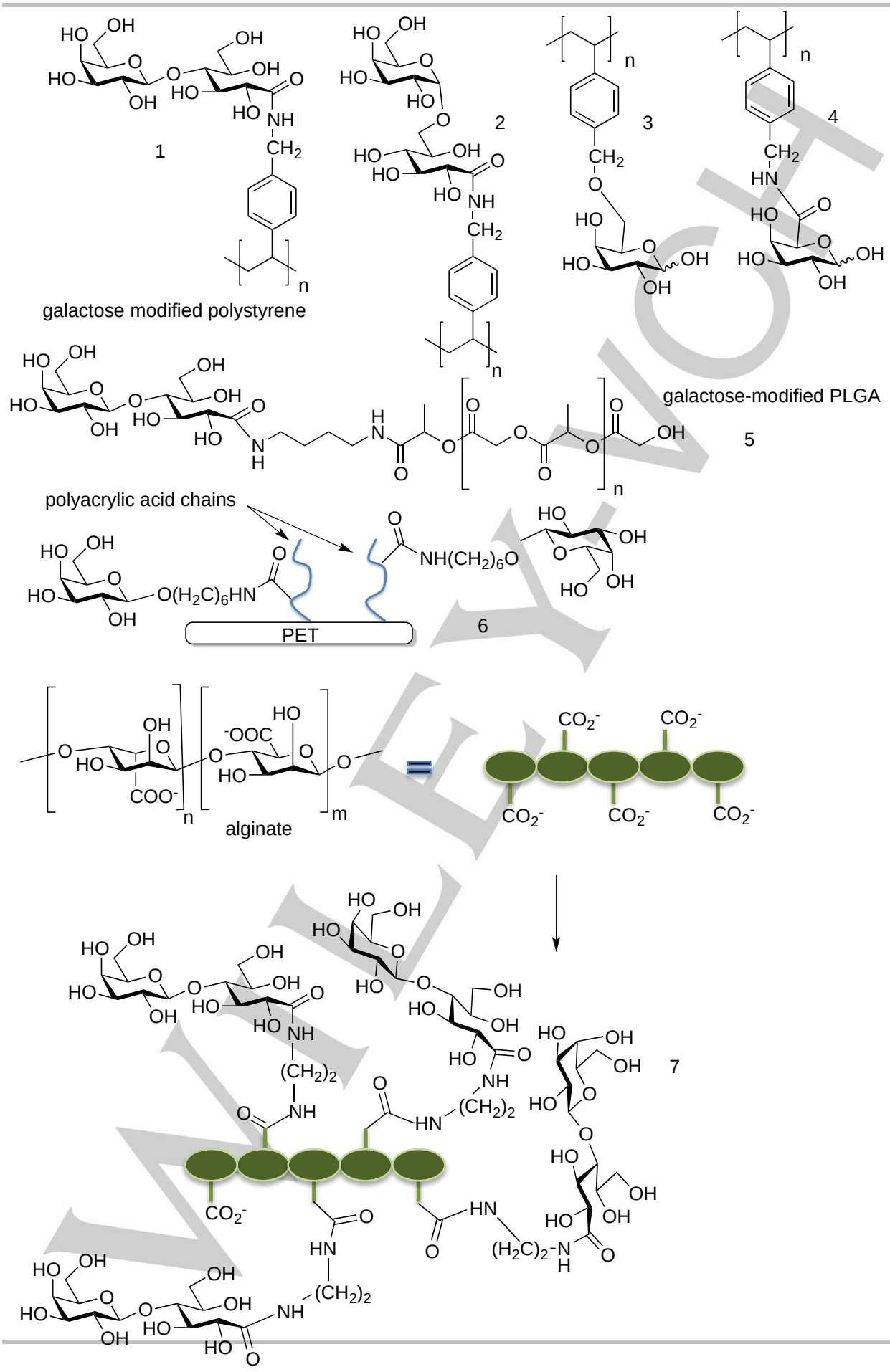
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FIGURE FOR THE FRONTISPICE