Chapter 23

Potential of Different Tissue Engineering Strategies in the Bladder Reconstruction

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Additional information is available at the end of the chapter

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1. Introduction

Organism's functions are provided by different biological apparatus and one of them is essential for maintaining the integrity of this system. Indeed activities of each organs lead to the cellular production of metabolites. These metabolic products are discharged into the blood system to be supported by the urinary apparatus. The purification of the blood is essential to preserve the homeostasis of the organism and the blood pressure. The upper urinary tract is composed of kidneys which filter the blood to evacuate the excessive water, ions and toxic metabolic products. Then, this mixture called terminal urine is transported in the lower urinary tract by the ureters. The lower urinary tract consists of the bladder which stocks the urine until its evacuation by the urethra. The terminal urine is cytotoxic because of its composition of nitrogenous and potential carcinogenic elements, also its pH which varies between 4.5 and 8.3 [1, 2]. Therefore, the storage of urine need to be safe, this is why the bladder possesses two specific characteristics.

Firstly, the bladder wall is watertight in order to prevent the urine from seeping through the different tissue layers and damage their structure. Secondly, the bladder is compliant in order to keep a low pressure during the urine filling, and prevent reflux towards the upper urinary tract which could lead to kidneys failure. Thereby, the bladder is able to adapt its capacity to the volume of the accumulated urine. These properties are entirely related to the nature and the structure of different tissues which compose the bladder wall (Figure 1). As shown in the figure 1, the tissue directly in contact with urine is the urothelium. This is an epithelium highly specialized regarding the watertight function and adaptation to large changes in urine volume.

This distensible property is assumed by the pseudo-stratified character of the urothelium, while the water tightness is provided by the most differentiated urothelial cells known as umbrella cells. These cells are endowed with very tight junctions in the apicolateral side, while a well-organized protein barrier, called uroplakin plaque, covers the luminal surface [3, 4]. The urothelium rest upon the lamina propria: an extracellular matrix which serves of nutritive and informative support for cells. The lamina propria is mostly made of collagen of type I and III [5, 6]. Another type of extracellular matrix is found at the lower level. It contains three organized smooth muscle cells bundles responsible for urine emptying because of their contractile property. This extracellular matrix is characterized by the presence of an elastic fibres network which allows the distension of the bladder and a low pressure during filing.

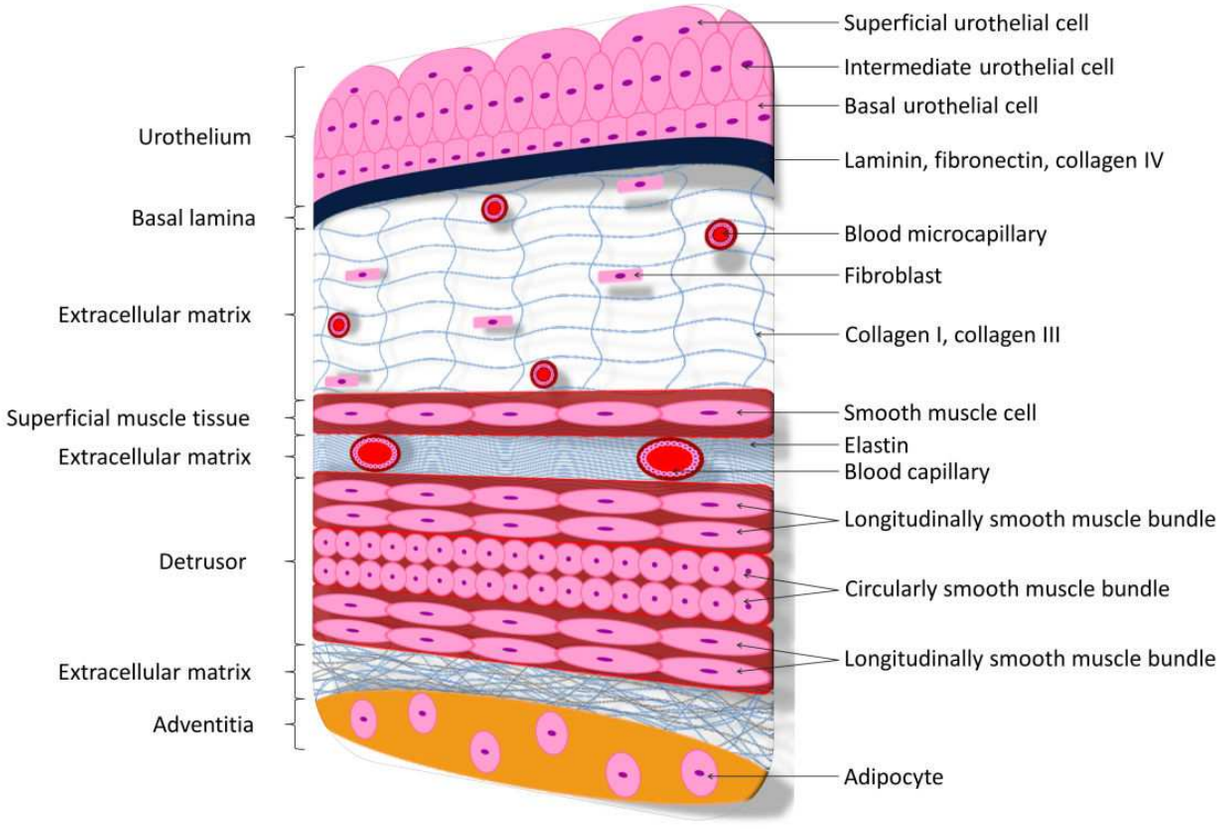

Figure 1. Scheme of bladder wall.

Every year 400 million person of all ages suffers from urinary disorders. Several congenital disorders such as bladder exstrophy or neurogenic bladder can affect the function of this organ. But there are also acquired bladder problems like traumatisms, inflammations, chronic interstitial cystitis and cancer which is the sixth most detected. All of these pathologies may require surgical augmentation or reconstruction of bladder wall to restore the storage capacity. The first application of a free tissue graft for bladder replacement was reported by Neuhof in 1917 [7], when fascia was used to augment bladders in dogs. Since that first report, diverse

methods have been proposed for this type of surgical intervention, but actually the gold standard is the bladder replacement/repair with autologous segment of the gastrointestinal tract, also named Enterocystoplasty [8, 9]. It has the advantage of being highly vascularized, promoting the survival of the graft. Unfortunately, this technique is associated with multiple short and long-term complications well documented (Table 1). The most frequent is metabolic disturbance, but mucus secretion, stone formation, bladder perforation and malignancy have also been found many years after enterocystoplasty [10-13]. These complications predominantly result from the difference between the absorption property of intestinal mucosa and the watertight function of the bladder epithelium, concerning the contact with urine.

	BLADDER
Pathologies	Congenital malformations Neurogenic bladders Bladder cancers Interstitial cystitis
Common treatment	Surgical bladder repair or reconstruction with intestinal segment. The choice of the segment depend mainly on age and medical history.
Associated complications	Intestinal dysfunctions Hypocontractility Hematuria, dysuria, urolithiasis Neoplasia Mucus production Metabolic imbalances [1]: delay of growth, and reduction of bone density in pediatric patients.

Table 1. Complications associated with current bladder treatment.

The lack of autologous tissue with similar properties to the native bladder is a limitation which led numerous research groups to develop alternative approaches. These last years, many fundamental knowledge concerning bladder cells and matrix have emerged, and have constituted an essential aid to the in vitro elaboration of various bladder models. This chapter will explain the different sources of cells used, the different type of engineered matrices, and the advanced concerning the techniques of in vitro culture. The emphasis will be placed on qualities and inconveniences of each approach, as well as the clinical potential of the engineered models.

2. Cellular source

Although the bladder is composed of many type of cells, the most harvested for vesical tissue-engineering is urothelial and smooth muscle cells. Urothelial cells are organized into three

layers which are anchored to the basal lamina. Basal cells, reside in the lower layer [14]. These progenitor cells develop themselves into intermediate cells, and differentiate into umbrella cells which are the most mature urothelial cells. The degree of urothelial differentiation is defined by the expression of specific proteins, such as keratins, claudins and uroplakins (Table 2) [15]. Smooth muscle cells have a fusiform shape and are assembled into bundles also organised into three layers. In the outer and inner layers, the smooth muscle bundles are oriented longitudinally, while those of the middle layer circularly. In each bundle, a single smooth muscle cell is innervated and action potential can propagated to neighboring cells in order to causes a coordinated contraction. The proliferation and differentiation of urothelial and smooth muscle cells are interdependent, because of factors released from these cells [16].

	UROTHELIAL CELLS			
Markers	basal	lower intermediate	upper intermediate	superficial
K5 - 10 - 17				
K13				
K14				
K7 - 8 - 18 - 19				
K20				
ZO1				Intercellular junctions
Cldn3				Intercellular junctions
Cldn4				Intercellular junctions
Cldn5				Intercellular junctions
Cldn7			Intercellular junctions	
Occldn			Intercellular junctions	
UPIa/Ib/II/IIIa				

Table 2. Markers of urothelial cells (white = no presence, light-blue = low presence, dark-blue = strong presence).

The importance to associate cells to a urological substitute was described by numerous studies [17-19]. These works showed that bladder substitute seeded with cells led to better in vivo regeneration, than the use of scaffold only. Cells help the graft integration; this is why the latest bladder substitutes are generally constituted by a combination of scaffold and cells.

2.1. Urological cells

Urothelial cells culture dates back to more than 30 years [20], and two major methods are developed for their extraction. The most former method is the explant technique. It consists to put a fragment of biopsy in nutritive medium, and let cells to migrate. Then, sequential action

of trypsin will permit to harvest different cells with distinct adhesion properties. The other way is the enzymatic technique which consists to detach the urothelium from basal lamina, undergoing matrix extracellular protease action (thermolysin, dispase, collagenase IV) [21, 22]. This last method is faster, led to a suitable yield and a good purity. Finally to obtain enough cells, an amplification stage must be engaged. Generally, epithelial medium is used and supplemented with serum or/and specific growth factor (e.g. epithelial growth factor).

Smooth muscle cells were first described in 1913. In the same manner of urothelial cells, the two approaches could be used for extraction [23]. In the case of enzymatic treatment, the collagenase will be used to digest the extracellular matrix, made of collagen I principally, to liberate these cells. In the amplification stage, it is important to know that the serum percentage could modify the phenotype (contractile or secretory) and the functional property (electrophysiological) of these cells [24-26].

Endothelial cells are more and more frequently extracted and used for bladder reconstruction. Indeed, after transplantation the graft need to be rapidly vascularized to survive in vivo. Endothelial cells could organize themselves into capillary or secrete angiogenic factors which could improve a certain inosculation between the substitute and the host vasculature [27]. Some teams harvest endothelial cells from human umbilical vein (HUVEC), what asks ethical question, and other achieve these cells extraction from bladder microcapillaries which appeared more physiological for the elaboration of the vesical substitute. In this last case, enzymatic treatment could be used and the harvested cells must be purified with beads coupled with a specific endothelial marker (e.g. PECAM-1) [21].

2.2. Stem cells

In some situation the bladder is too affected and no healthy cells could be harvested to elaborate a tissue-engineering substitute. So, stem cells could represent a serious alternative and major avenue in the regenerative medicine. These cells are characterised by their capacity to maintain themselves by symmetrical division. But in a second phase, asymmetrical division occurs and leads to a daughter stem cell and a daughter differentiated cell. This last event makes difficult the in vitro preservation of stem cells and the constitution of an usable stock.

Embryonic stem cells are pluripotent and therefore can evolve into cells of all three embryonic layers (ectoderm, mesoderm and endoderm). The method of culture to enable specific differentiation pathways are not yet discovered, but the in vivo benefits have already been shown [28-29]. Unfortunately, their aptitudes to initiate teratoma, the need of genetic donor-host match to avoid immune-rejection and their potential illegal and non-ethical character compromise their clinical use.

Autologous stem cells could resolve these limitations. They present a low possibility of tumor malignancy and an exact histocompatibility. These cells niche within many adult tissues, in order to maintain homeostasis during tissue turnover and repair. However, they present lower proliferation capacity and plasticity which restricted their use.

Induced pluripotent stem cells appeared in 2006 and consist of reversion of differentiated cells into stem cells [30]. The reprogramming of adult cells requires the introduction of specific

transcription factors (Oct4, Sox2, Klf4 and Myc), which allow to acquire differentiation potential comparable to the embryonic stem cells [31, 32]. This ethical approach provides an easy and important cell sources for clinical application. However, even if a major risk of cancer has been corrected [33], epigenetic changes not yet entirely documented.

3. Nature of scaffolds

Once the cells have been obtained, they must be seeded onto a support (Table 3). The scaffolds are not only a physical support, but also supplier of biochemical information. It is difficult to encourage an appropriate in vitro cellular behaviour because spatial and temporal evolutions occurred during organogenesis. The complexity of extracellular matrix sequentially increases from morula to blastocyst stage [34, 35], and the signalisation between cells and extracellular matrix proteins change in a dynamic way [36]. In the present state of our knowledge, laminin is firstly synthetized to allow cellular adherence, while collagen IV and fibronectin appear more latter to initiate the cellular migration [37]. In a second phase, these extracellular matrix proteins form an organized matrix, the basal lamina, which is essential for the development of specialized tissue like epithelium [38]. Thus, tissue engineering must tend to create a controlled cellular microenvironment, taking into account the physiological process, to reach the experimental organogenesis goal. In another hand, scaffolds must offer comparable physical properties and have the capacity to confront the same mechanical strain. In the case of vesical repair, the substitute must combine resistance with elasticity to adapt itself to cyclic pressure caused by bladder filling and emptying. The scaffold biocompatibility is the starting point of material choice, and many products have been experimented with a clinical application point of view.

STEPS	DESCRIPTION
Harvesting bladder cells	Very small biopsy is taken. The different tissues are separated. Cells from each tissue are collected.
Cells amplification	Protocols exempt of biological risks. Using serum must be from the patient. Using growth factors must be recombinant.
Assessing cells into a support	Seeded cells must be functional and located at their physiological area.
Surgical implantation	The substitute must be functional before the bladder repair.

Table 3. General procedure in bladder reconstruction field.

3.1. Natural matrices

Autologous tissues have been tested for bladder repair before the standard use of intestinal segment in the beginning of the eighties [1]. The skin, the omentum, the stomach, the pericardium have been used with very limited success [39-42]. Indeed, the epithelium of these tissues is not specialized in watertight function. So, the direct contact between the non-urological tissues and the toxic urine leads mainly to the formation of fibrosis and the contraction of the graft [43, 44].

Acellular tissue matrices represent a growing interest in the urological regenerative medicine. They are prepared from native tissue with a decellularisation and sterilisation process [45]. The purpose is to abolish immunological potential by discarding cells with physical, enzymatic or chemical protocols [46]. This materials offer the advantages of mechanical and biochemical environment ideal for the cellular recognition. Their matrix architecture is relatively preserved and the physiological organization of extracellular proteins can generate appropriate signalling to induce a suitable cellular behaviour [47]. Unfortunately, the decellularisation and sterilisation protocols include offensive biophysical and biochemical elements (temperature, pH, ionic detergents) which can denature extracellular matrix proteins and damage the physiological environment [48]. Compared to other scaffolds, the ability to provide a nutritive supply by neovascularization after the graft, and therefore to promote the graft survival constitutes an attractive advantage which justifies the choice of acellular matrices [49, 50]. Small intestinal submucosa (SIS) [51] and bladder acellular matrix graft (BAMG) [52, 53] are the most tested for bladder reconstruction in animal models. Microscopic analyses generally show a relative cellular organization, probably due to the presence of growth factors [54]. But functional tests such as watertightness or compliance are very seldom evaluated.

Concerning SIS, studies are conflicting. Some experiments describe the benefit to use SIS with contractility testing and immunostaining analysis [18], while other report fibrotic scarring observations and contraction during in vivo bladder augmentation in canine model [55]. Histologically, the urothelial and smooth muscle cells seeding led to a suitable adherence but without cellular maturation. This lack of regenerative potential could be caused by an absence of the appropriate extracellular proteins-cells communications, due to the fact that intestinal and bladder matrices are not similar [56]. It is known that extracellular matrix composition and structure presented by the bladder basal lamina have an impact on urothelial cells behaviour [57]. This is why bladder acellular matrix graft (BAMG) has been also tested. However, porcine bladder augmentation with a seeded BAMG demonstrated a local cells infiltration which remained limited in the periphery [58]. The insufficient cellular organization resulted in calcification process within the graft and its shrinkage. This incomplete cellular migration within the BAMG could be attributed to the decellularisation treatment, because it was reported that laminin and fibronectin are not preserved [59]. Thus, even if urological cells are placed into a familiar environment, the alteration of matrix proteins and the removal of intrinsic growth factors do not permit an optimal cellular signalisation [60]. Moreover, the disruption of the extracellular matrix compromises the specific architecture and leads to the loss of mechanicals properties, such as elasticity or resistance [61]. Optimization of these

protocols is in progress and the damages caused by the preparation of decellularised tissue tend to be corrected [62, 63].

Intestinal segment and bladder wall are richly vascularized tissues, and this specificity is not negligible for the survival of the graft. However some parameters limit the clinical use of BAMG and SIS such as their xenogeneic origin and the associated risks of contamination by pathogens. Generally, the matrix proteins are highly conserved between the different species and seem to be non-immunogenic but an epitope, which is present in many species except human and a category of monkeys, was found in marketed SIS extracellular matrix [64]. Numerous postoperative inflammations have been reported [65] and in vitro DNA residues have been detected [66]. Because of their biological nature, the acellular tissues constitute a great potential for bladder reconstruction but the protocols of preparation must be improve to better preserve the extracellular matrix composition/architecture and to remove effectively any cellular fragments [67].

3.2. Synthetic scaffolds

Synthetic polymers are made of chemically assembled macromolecules and could have different physical properties (thermoplastics, thermo-hardenings and elastomers). The use of synthetic scaffolds, supported by the capacity to deliver any three-dimensional shape at low-price, would allow to overcome the lack of native tissue available for transplantation. Each characteristic of this type of material can be controlled. The hydrolysis degradation of synthetic substitutes is important in the context of organ repair. The material used for bladder reconstruction must remain stable until the organization of seeded cells and the migration of surrounding cells. But during the terminal tissue remodelling, the substitute degradation must follow the tissue regeneration. The degradation rate of synthetic materials can be determinate by the molecular weight and the nature of biopolymer used for copolymerisation. Then the degraded fragments can be treated by the metabolic pathways [68-70]. The size of pores can also be modulated in order to influence the cellular migration, vascular invasion, and diffusion of nutrients, waste and oxygen [71, 72]. The cell adherence and growth can be enhanced by synthetic support, as shown for the reconstruction of different damaged tissues [73]. But in the case of bladder substitute, the in vitro evolution of seeded urologic cells into a mature cellular tissue has not yet demonstrated. There are fundamental differences between synthetic and biological molecules. The size range of fibers, their biophysical and biochemical properties are not comparable, and the challenge for these supports is to enhance a suitable interaction between cells and synthetic microenvironment, in spite of the dissimilarities existing with the physiological context.

Silicone, polyvinyl sponge and teflon, have been firstly tested for bladder reconstruction with the help of synthetic materials [74-78]. The advantage was to construct neobladders in a reproducible way, but the poor cytocompatibility, the lack of vascularization and their immunological potential led to significant complications. Because of the absence of cellular development, and urothelial cells particularly, the direct contact with urine caused the formation of fibrosis and contraction of the alloplastic graft.

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The more recent synthetic polymers including poly(ethylene glycol) (PEG), poly l-lactic acid (PLLA), poly(lactic-co- glycolic acid) (PLGA), poly(ε-caprolactone) (PCL), and polyurethane (PU), are used to pursue the bladder tissue-engineering research [79]. Few successes have been reported, but like in the case of acellular tissue matrices, it has been shown that the cell seeding play an important role concerning the graft integration [19]. This experience of canine bladder reconstruction have been led at mid-term with PGA scaffold coated with PLGA, and contrarily to the seeded synthetic substitute, contracture and inflammatory response have been observed with free scaffold one month post-transplantation. In spite of association of urothelial cells with a high proliferative capacity when urothelium is damaged [80], the surrounding cells of the host do not migrate in the whole surface of tissue-engineered bladder. The only one microscopic result demonstrated a well development of an urothelial tissue onto the seeded scaffold, with the presence of muscle bundles, but no analysis was led to assess the degree of urothelial maturation or the contractility function of smooth muscle cells. The potential of synthetic materials to support urothelial differentiation have not been proven yet. The same team have combined collagen with their PGA scaffold to lead bladder augmentation in patients with end-stage bladder disease [81]. The strategy is to supply a three-dimensional shape and mechanical resistance with the help of PGA, and to induce a cellular signal with the help of the collagen. It was shown that the biological activity provided by collagen improve the cellular propagation and development of seeded urothelial and smooth muscle cells. Additionally, omentum was used as a vascularization bed to support the graft survival [82]. Even if significant improvement of vesical capacity has been shown for only one of the seven patients, great strategic progresses have been made to initiate the elaboration of a composite scaffold able to communicate with cells and enhance their appropriate and terminal development. The scale of cellular environment and signalisation induced by specific protein sequences have been taking into account with the advent of biotechnologies.

3.3. Nanobiotechnologies

The nanotechnologies, which emerged in the last decade of the twentieth century, are defined by specific functions induced by the nanoscale dimension or the nanoscale organization of a material [83]. This characteristic could permit to overcome the problems encountered with the classic synthetic substitutes made of micrometer scale elements. The interest to include nanometer elements in the composition of synthetic scaffolds is to reproduce the scale of the size of native extracellular matrix proteins, and better manage cellular behaviour [84]. Recent researches demonstrated the effect of roughness surface, comparable with the roughness of the native bladder, on smooth muscle cells adhesion and urothelial cells development [85-87]. On another hand, the surface of synthetic materials could be modified with different chemistry, topography or roughness parameters, to improve the interaction with proteins. Thus, extracellular matrix proteins could be added to the scaffold in order to enhance the cellular interaction and to tend toward natural signalisation. Recent publication reported that vitronectin adsorption is improved by 20% if the roughness of synthetic surface is augmented with nanotechnology process, compared to nanosmooth surface [88]. A concrete example is illustrated by the increase of fibronectin adsorption onto synthetic material, because of the

augmentation of roughness surface by adding carbon nanotubes and without changing the chemical properties.

Smooth muscle cells phenotype is affected by the nanometer topography of synthetic surfaces. This phenomenon was illustrated by recent works. One of them reported the elaboration of PLGA/PU materials with different feature dimensions. It is appeared that more the features size is comparable to the nanometer scale, like the extracellular matrix proteins, more the adhesion of bladder smooth muscle cells is improved [85]. A new chemically etching technique, which breaks ester and ether bonds via NaOH and HNO3 treatments, was tested by the same team to convert synthetic surface from micrometer to nanometer scale [89]. It was shown that this chemical treatment can generate nanoscale features on various synthetic surfaces (PLGA, PCL, PU), and then enhances the functions of bladder smooth muscle cells, compared to the conventional nanosmooth polymers. It is noted that micron, submicron and nanostructured polymers was generated with this same chemical protocol, and the development of bladder smooth muscle cells have been improved on the nanostructured surface, independently of a chemical action. So, any type of synthetic scaffold could be nano-engineered and promote bladder smooth muscle cell functions, such as elastin and collagen secretion [86]. Further studies demonstrated that the direct action of nanometer and submicron scale surface features is to promote the adsorption of proteins from serum present in culture media. Therefore it seems that the bladder smooth muscle cells behaviour is more directly improved by this serum proteins coating rather than the synthetic nanostructured environment [90, 91]. These observations lead us to believe that the next generation of synthetic substitute could be grafted with soluble proteins in order to ameliorate the cellular signalisation.

Urothelial cells growth is also affected by the roughness of synthetic surfaces [87]. With nanometer rough synthetic surface, results show the improvement of adhesion and proliferation of urothelial cells, and also the reduction of calcium stones, often noticed with conventional synthetic materials. In vivo studies were performed in rodent model and confirmed the benefits showed with bladder smooth muscle cells. But nanostructured synthetic scaffold did not resist the contact with urine, leading to post-graft bladder leaks [92]. Further optimizations must be proposed to induce cellular differentiation. Fibronectin is recognized to induce cell migration, laminin for its adherence potential, therefore synthetic scaffolds will be completed with bioactive nano-elements. Protein sequences implicated in cellular signalisation will be used such as RGD peptide, a cell adhesive integrin-binding peptide found in most of extracellular matrix protein [93]. The development and maturation of seeded cells must be evaluated at longer term, while in vivo testing will occurred throughout animal models more comparable to human (the porcine urologic system is the nearest of human [94]).

4. The self-assembly method

The natural and synthetic materials have their own characteristics (Table 4), specific bioactive elements for the first and processing reproducibility for the second. The advances deserve a particular attention even if these experimental scaffolds could not be recommended at that

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time for clinical application. Acellular matrices present the risk of incomplete decellularisation and variability of biological activity. While synthetic materials are too far from the composition and architecture of physiological extracellular matrix. But one of the common disadvantages of these two models is the possibility of immunological response in vivo and therefore a graft rejection.

BIOMATERIALS	CHARACTERISTICS
Non autologous natural matrices	+ Biologically organized tissue. + Specific growth factors reservoir. + Augmentation of tissue availability. - Damaged architecture of matrix. - Presence of cellular fragments. - Risk of xenogeneic contamination.
Synthetic matrices	+ Control of physical properties. + Biodegradable. + Evolution towards nanoscale. - The deficiency of biological signalization. - Cicatrization with the host's tissue. - The cost.
Autologous matrix	+ Limitation of immunoreaction. + Self-assembled tissue. + Evolution towards physiological stimuli. - Lack of *in vivo* investigation.

Table 4. Types of engineered bladder substitute.

Autologous and functional substitute represents the ideal alternative to avoid immunosuppressive therapy and to enhance the in vivo regeneration. The tissue-engineering field proposes a new approach to attain these objectives: the self-assembly method [95]. This technique is based on the use of the own cells of patient and their capacity to differentiate in vitro in order to form a mature tissue. At the Laboratoire d'organogénèse expérimentale (LOEX) many autologous substitutes are elaborated with controlled culture conditions and without the help of exogenous biomaterials. Human cornea [96], psoriatic skin model [97], microvascularized tissue-engineered skin substitute [98] has been developed for clinical repair or pharmacological investigations. Based on this method, an autologous vesical tissue was elaborated with a view to future clinical bladder repair/augmentation.

4.1. Autologous vesical substitute

A minimally invasive preoperative technique was developed to extract the bladder cells from the same small biopsy. Enzymatic method allows the harvesting of bladder cells with high purity [21]. Fibroblast, urothelial, endothelial and smooth muscle cells can be grown in the

laboratory setting. Appropriate growth factors (e.g. epidermal growth factor for urothelial proliferation or vascular endothelial growth factor for endothelial cells) permit a good cellular expansion and the constitution of sufficient cellular bank.

The preparation of engineered vesical tissue begins with the elaboration of matrix support [99]. In presence of ascorbic acid [100, 101], fibroblasts have the ability to synthesize and assemble their own collagen fibers, and form an extracellular matrix layer which could be manipulated. The autologous matrix layers are superimposed to provide a sufficient mechanical resistance and to create a three-dimensional biological environment. It is known that mesenchymal-epithelial interactions play critical role in tissue development [102-105]. In the urological tract, mesenchyme regulates epithelial maturation and functional activities, while epithelium also contributes to the mesenchymal cells differentiation [106]. This is why the self-assembled scaffold is made of a specifically organized extracellular matrix where cells can develop themselves with appropriate biological signalling. Urothelial cells are seeded on the top of fused matrix layers and cultivated until they have proliferated on the whole surface, then we induced epithelial maturation with the use of the culture at air/liquid interface (Figure 2).

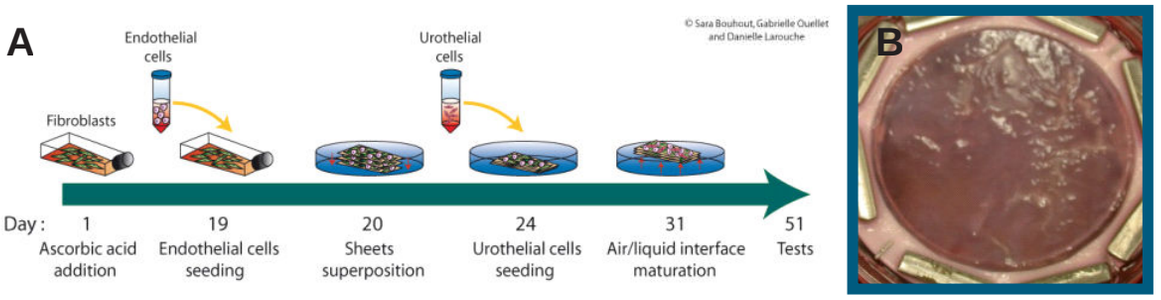

Figure 2. The self-assembly method (A) used to elaborate the engineered bladder tissue (B).

Masson's trichrome staining displayed a homogenous distribution of collagen I and the covering of stratified urothelium was roughly similar to a native vesical mucosa. Cytokeratin 8/18 staining confirmed the well widespread of a stratified urothelium, and claudine-4 staining demonstrated the presence of tight junctions which are important to avoid urine infiltration. More interestingly, the permeability evaluation at urea was performed on the reconstructed vesical tissues and their watertightness profile is comparable to the native bladder. Conversely, the permeability test realized on self-assembled matrix, devoid of reconstructed urothelium, showed a fast diffusion of urea. This result supports the necessity to have a mature urothelium, in order to avoid urine extravasation, followed by in vivo necrosis and fibrosis of tissues. An attention was paid to the endothelialization of the substitute in order to encourage the post-graft survival. LOEX laboratory obtained a rapid inosculation between a capillary-like network of reconstructed skin and the host vasculature [6]. Thus endothelial cells, harvested from bladder microcapillaries, can be added before the seeding of urothelial cells, and cultured in order to form microcapillary-like network within the self-assembled substitute. The endothelialized vesical substitute displayed an also good watertightness profile than the native bladder, and mechanical properties were acceptable to allow suturing. The potential limit of

this method is the incertitude to dispose of healthy bladder cells, especially in a case of end-stage disease. This is why the question concerning the getting of an appropriate source of cells remains in reflection, but in some case, urological cells could be harvested from upper segment of urethra because of its sharing of the same embryologic origin with the bladder [107]. The self-assembled vesical model still must to be completed and tested in vivo, but its autologous character and its efficiency as a barrier to urea are essential properties to tend toward an ideal substitute for bladder augmentation.

4.2. Culture conditions

In regenerative medicine domain, the goal of tissue engineering is to deliver a functional substitute. It consists in obtaining specific cells with sufficient purity and quantity, and inducing their differentiation in order to ensure their biological role. Self-assembled extracellular matrix provides both a physical and informative support for urological cells, but the control of cellular proliferation/differentiation balance deserve to be better taking into account. Several studies reported an altered maturation of urothelial cells in vitro, particularly a defect of uroplakins synthesis [108] and their incomplete assembly at the apical surface of urothelial cells [109, 110]. The pathway of differentiation is induced by defined intracellular signals. Transcription factors such as Fox-A1, PPARγ and RXR, have been identified as being involved in the expression of proteins implicated in the urothelial functions [111]. The bladder is subjected to different pressure during the emptying/filling cycle, and the cells are constantly exposed to mechanical stresses (e.g. hydrodynamic strength) [112]. It was shown that mechanical stress acts on survival, migration and proliferation of bladder cells [113-115]. More accurately, cyclic hydrostatic pressure seems to promote uroplakin trafficking/maturation [116-118], and to influence the growth of bladder smooth muscle cells by activating Rac1 signaling pathway [119], or their proliferation via the PI3K activation [120]. Even if the molecular mechanism acting under cyclic pressure remains not elucidated, the conviction that physiological stimulations would be required for the differentiation of bladder cells cultured in vitro is more and more evocated in the literature [121-123].

In the beginning, mechanical stimuli were included in cellular culture protocols and interactions between cells or between cells and extracellular matrix proteins were analyzed [124, 125], but physiological conditions were not yet investigated. In 2008, the mimic of bladder filling and emptying was tested through a bioreactor which applies hydrostatic pressure waves in a cyclic way [126]. Porcine BAMG was used and the expression of extracellular matrix proteins was more elevated under dynamic condition. This result is encouraging for acellular matrix application. Indeed, acellularisation treatment deteriorates the matrix architecture and the obtained tissue after treatment had a too high porosity [127]. Because of its property to support cell proliferation and migration, hyaluronic acid could be used in static regime to decrease the porosity observed after the acellularisation process [128], but this step could be replaced by placing the reconstructed substitute under physical stress. The urothelial cells were also influenced since mechanical stimulations induced the increasing of uroplakin II expression. The effects of physical strain must be studied in more details because gene expression does not constitute sufficient information, since the protein functionality depends on its

complete synthesis, its good folding and its appropriate localization. But these preliminary results encourage the integration of mechanical phase in the process of engineered bladder. This is why we designed a bioreactor which is scheduled to reproduce the physiological intravesical pressures at the fetal stage, and replace the air/liquid phase used for our self-assembled model. Briefly, our self-assembled vesical tissue is placed between two chambers, with urothelial side face to the pressure chamber. To mimic the low pressure maintained during filling phase, 5 cm H2O is applied during few hours. In the last hour the pressure slowly increase until 15 cm H2O, and then decline quickly to zero in a few seconds in order to simulate the voiding and complete the urination cycle. Compared to static condition, short-term dynamic culture significantly improves the urothelial development and the watertightness profile of the self-assembled substitute [129]. These results are in conformity with outcomes related in our dynamical engineered urethra which displayed an increase of uroplakins immunostaining at urothelial cells surface [130]. Whatever the substitute model, the mechanical stimulations must take more importance within protocols of in vitro bladder reconstruction. When must the dynamic phase intervene? How long time must it intervene? Should the pressure cycle follow a constant scheme or must it evolved during the process? Better understanding of bladder cells mechanotransduction may ameliorate the setting up of a dynamical environment appropriate to the reconstruction of a mature and functional vesical tissue.

5. Conclusion

Bladder exposition to diverse pathologies could jeopardize its function of elastic and watertight reservoir. To date, the clinical technique for bladder repair is associated to a high level of morbidity. Based on the well documented post-operative complications, it is appear that the ideal bladder substitute must combine the compliance conferred by the nature and architecture of the matrix support, with a urinary barrier provided by the differentiation degree of urothelium. Natural and synthetic scaffolds were investigated to reproduce the bladder abilities and some successes were furthered the urological tissue-engineering domain. But due to their poor mechanical stability, immune responses, and incomplete cellular maturation, these models remain insufficiently developed to be used in clinical application. At present, teams which support the acellularised or polymeric substitute are working on the next generation of engineered bladder model. For example, nanodimensional surface features would be included in order to imitate the nanometer topography of native tissue, and therefore, to enhance interactions between bladder cells and the proposed environment. Among all biotechnologies, the self-assembly method proves to be a promising approach to elaborate a vesical substitute comparable to the structure and function of native tissue. The good watertightness of reconstructed mucosa and its autologous character will permit a suitable integration in vivo, and promote the cellular expansion. The application of appropriate culture method, such as dynamical regime, will lead to the maturation of the reconstructed connective tissue and its urothelium. The capacity of the self-assembled tissue to be pre-endothelialized might avoid the necrosis of the graft attributed to the lack of synchronized neovascularization. Another aspect which is rarely taking into account is the capacity for a graft to growth with

pediatric patient. The self-assembled tissue is made of autologous cells only, and constitutes a serious alternative in the urological tissue-engineering field. Research continues its efforts to optimize the different reconstructed substitutes, and agree the necessity to evaluate them at long-term through bladder with specific dysfunctions.

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